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CHANGES IN THE POLYUNSATURATED FATTY ACID CONTENT OF POTATO TUBERS DURING GROWTH, MATURATION AND STORAGE. J. H. SCHWARTZ, R. E. LADE & W. L. PORTER. *J. Food Sci.* **33**, 115–118 (1968)—During storage linoleic and linolenic were almost the only polyunsaturated acids present, but during growth and maturation considerable amounts of unidentified polyunsaturated acids were found. The percentage of polyunsaturated acids in the dry weight of tuber decreased to a low value near harvest time and remained near this value throughout the 6.3-month storage period, except that the value in Pontiacs stored 19 days was somewhat high. The percent of polyunsaturates in the total fatty acid fraction also dropped to a low value during growth and maturation but increased somewhat during storage.

IDENTIFYING NUCLEOTIDIC MATERIALS RELEASED BY FERMENTING BREWER'S YEAST. T. C. LEE & M. J. LEWIS. *J. Food Sci.* **33**, 119–123 (1968)—During fermentation a brewer's yeast released into the medium substantial amounts of materials absorbing ultra-violet light at 260 $m\mu$. These materials were separated by chromatography and identified by further chromatography and spectrophotometry. Nine nucleotides, five free bases (purines and pyrimidines) and four nucleosides were identified in the fermented medium and in the intracellular pool of the yeast. Some of these released materials may be of consequence in the fermented beverage industry since they were not reabsorbed by yeast and can be expected to persist to the finished product.

MECHANISM OF RELEASE OF NUCLEOTIDIC MATERIAL BY FERMENTING BREWER'S YEAST. T. C. LEE & M. J. LEWIS. *J. Food Sci.* **33**, 124–128 (1968)—When a strain of brewer's yeast grown under suitable conditions was suspended in a solution of fermentable sugar, nucleotides and other U.V.-absorbing materials were rapidly released from the cells. The extent of release depended on the pH of the medium, the temperature, the concentration for fermentable sugar and the presence of membrane-protecting (Ca^{++} or Mg^{++}) or membrane-damaging (butanol, detergent) reagents.

IDENTIFICATION OF SOME SUGARS AND MANNITOL IN CELERY. R. BECKER. *J. Food Sci.* **33**, 128–130 (1968)—The carbohydrate content of celery petioles was determined using paper chromatographic techniques. Sucrose, glucose, fructose and mannitol were identified and quantitatively determined. Mannitol crystals were isolated. Sugars chromatographed with solvents containing boric acid showed characteristic stabilities to indicators.

SEROLOGICAL IDENTIFICATION OF ANIMAL PROTEINS. 1. Mode of Injection and Protein Extracts for Antibody Production. M. O. WARNECKE & R. L. SAFFLE. *J. Food Sci.* **33**, 131–135 (1968)—Specific antibodies were developed against skeletal muscle from horse, pork, lamb and beef. The antigenic protein material evaluated for antibody production included actomyosin, serum-alum precipitate, muscle extract-alum precipitate, saline extract of muscle and freeze-dried water extract of muscle. Of the antigenic protein material and route of injection evaluated, the intramuscular injection of 150 mg of freeze-dried water extract of muscle with Freund complete adjuvant resulted in the highest titers which were observed for at least 120 days.

IONIZING RADIATION EFFECTS ON STARCH AS SHOWN BY STAUDINGER INDEX AND DIFFERENTIAL THERMAL ANALYSIS. V. SAINI. *J. Food Sci.* **33**, 136–138 (1968)—Corn starch in the form of raw granules at commercial moisture was irradiated at two levels i.e. 3×10^6 and 6×10^6 rad from a Co^{60} source. Viscosity determinations of starch solution diluted with distilled water exhibited the ion charge effect generally observed in other macromolecules. The Staudinger indices of unirradiated, irradiated at 3×10^6 rad and 6×10^6 rad were 42, 22 and 16 respectively, which were an indication of depolymerization of starch macromolecules with increasing irradiation. The differential thermal analysis of the three samples also showed the depolymerization of the polymer with irradiation.

THERMAL DEGRADATION OF BLACK RASPBERRY ANTHOCYANIN PIGMENTS IN MODEL SYSTEMS. G. DARAVINGAS & R. F. CAIN. *J. Food Sci.* **33**, 138–142 (1968)—The degradation of the major anthocyanin component (cyanidin-3-diglucoside), the total isolated pigments and the pigment in the natural berry juice was retarded as the pH decreased. Under the same conditions, cyanidin was much more unstable than any of the anthocyanin containing systems. In all cases studied, replacement of the oxygen atmosphere with nitrogen enhanced pigment stability. The rate constants for the thermal degradation of cyanidin-3-diglucoside at various pH levels under oxygen and nitrogen were determined. The sugars studied accelerated pigment destruction to the same extent.

FREE AMINO ACIDS IN HAM MUSCLE DURING SUCCESSIVE AGING PERIODS AND THEIR RELATION TO FLAVOR. G. R. McCAIN, T. N. BLUMER, H. B. CRAIG & R. G. STEEL. *J. Food Sci.* **33**, 142–146 (1968)—The free amino acids and total ninhydrin positive material (NPM) in a 1% picric acid extract from dry-cured hams were measured after six different periods of aging. Correlation coefficients were calculated between amino acid values and taste panel scores. Significant ($P < .05$) increases were observed for NPM, serine, glutamic acid, threonine, leucine and isoleucine (not separated), valine, phenylalanine, proline, tyrosine, alanine, glycine, and histidine during successive aging periods.

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BROWNING AND ASSOCIATED PROPERTIES OF PORCINE MUSCLE. J. A. BOWERS, D. L. HARRISON & D. H. KROPF. *J. Food Sci.* **33**, 147–151 (1968)—Half carcasses of untreated, sugar-fed, and exercised pigs were chilled (30° and 42°F). Duroc muscle usually exhibited less moisture, higher "quality" scores and greater ether extract and browning than Poland China muscle. Color, firmness, glycogen, reducing sugar, and pH were similar for both breeds. Sugar-feeding produced muscle highest in glycogen, reducing sugar, and browning. Exercise produced the highest pH; no treatment gave intermediate values for most factors studied. Regression analyses and partial correlation coefficients indicated that browning increased as ether extract and reducing sugar increased.

GLYCOLYTIC INTERMEDIATES AND CO-FACTORS IN "FAST"-AND "SLOW-GLYCOLYZING" MUSCLES OF THE PIG. L. L. KASTENSCHMIDT, W. G. HOEKSTRA and E. J. BRISKEY. *J. Food Sci.* **33**, 151–158 (1968)—The Longissimus dorsi muscles from Chester White, Hampshire and Poland China animals were used to establish certain differences in metabolic intermediate patterns between muscles with "fast" and "slow" rates of post-mortem glycolysis. Metabolic intermediate patterns were consistent with the concept that phosphorylase is the primary control site of post-mortem glycolysis. Adenine nucleotide levels appeared to be the primary regulatory factors for phosphorylase. The phosphofructokinase and pyruvic kinase enzymes were also involved in post-mortem glycolytic control.

THE NON-SAPONIFIABLE CONSTITUENTS OF LETTUCE. F. KNAPP, R. AEXEL & H. J. NICHOLAS. *J. Food Sci.* **33**, 159–162 (1968)—By means of thin-layer chromatography, gas-liquid chromatography and chemical analyses the following substances were identified in dried Iceberg lettuce (*Lactuca sativa* L.): cervl alcohol, β-sitosterol, stigmasterol, campesterol and the glycosides of the latter three sterols. An unidentified substance, probably a sterol, was detected by gas-liquid chromatography. A mixture of triterpenes identified as containing β-amyrin, α-amyrin and ψ-taraxasterol was also found.

CHEMICAL MODIFICATION OF THE EGG WHITE WITH 3,3-DIMETHYLGLUTARIC ANHYDRIDE. S. K. GANDHI, J. R. SCHULTZ, F. W. BOUGHEY & R. H. FORSYTHE. *J. Food Sci.* **33**, 163–169 (1968)—Egg white was reacted with 3,3-dimethylglutaric anhydride (DMGA) under mild reaction conditions. Eighty % of the free amino groups and 25% of the sulfhydryls reacted at 60 mol DMGA/mol egg white protein (assuming egg white to have an average molecular weight of 50,000). Electrophoresis indicated significant changes of net protein charges, particularly on lysozyme which migrates anodically in six separate bands at higher DMGA levels. No molecular aggregation is suggested by ultracentrifugation. Foam formation is not seriously altered, but the heat coagulation properties, as evidenced by angel cake failures, are affected.

DRY LAYER PERMEABILITY AND FREEZE-DRYING RATES IN CONCENTRATED FLUID SYSTEMS. D. G. QUAST & M. KAREL. *J. Food Sci.* **33**, 170–175 (1968)—Permeability was higher for slowly frozen samples of coffee and of a model system, than for rapidly frozen samples. A surface layer of very low permeability was observed in all samples frozen without agitation. Permeabilities obtained in steady state measurements correlated with permeabilities calculated from freeze-drying rates.

FATTY ACID COMPOSITION OF THE INNER AND OUTER LAYERS OF PORCINE BACKFAT AS AFFECTED BY ENERGY LEVEL, SEX AND SIRE. D. E. KOCH, A. F. PARR & R. A. MERKEL. *J. Food Sci.* **33**, 176–180 (1968)—In two experiments, the inner layer of backfat consistently contained more saturated fatty acids, primarily accounted for by stearic acid, and contained less palmitoleic, oleic and linoleic acids than the outer layer. None of the variables in either experiment significantly ($P > .05$) affected the differences between layers. In Experiment 1, energy levels below 80% of full feed significantly ($P < .01$) decreased stearic acid content and increased linoleic acid content. In Experiment 2 the fatty acid composition of backfat from pigs fed 80% of full feed did not differ significantly ($P > .05$) from full-fed pigs.

A STUDY OF CERTAIN PROPERTIES OF MYOSIN FROM SKELETAL MUSCLE. D. W. QUASS & E. J. BRISKEY. *J. Food Sci.* **33**, 180–187 (1968)—Myosin preparations were found to be pure (by ultracentrifugation, Sephadex separation and superprecipitation tests) and stable with normal values for SH groups. The Ca^{++}-activated ATPase activities of myosin extracted from PSE Poland China pigs were significantly greater than those from Chester White pigs and normal Poland China pigs. EDTA-activated ATPase activities were greater in myosin from PSE Poland China than in normal Poland China.

COMPOSITION OF BOVINE MUSCLE LIPIDS AT VARIOUS CARCASS LOCATIONS. P. W. O'KEEFE, G. H. WELLINGTON, L. R. MATTICK & J. R. STOUFFER. *J. Food Sci.* **33**, 188–192 (1968)—Bovine intramuscular lipids extracted from the semitendinosus triceps brachii and longissimus dorsi muscles were fractionated into neutral fats and phospholipids. There were significantly lower contents of total fat and neutral fat in the semitendinosus as compared to the other two muscles. The phospholipids were present in all three muscles at a level of approximately 500 mg per 100 g of tissue. There was significantly more C14:0 in the longissimus dorsi neutral fat fractions than in the semitendinosus neutral fat fractions.

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KINETICS OF THE ENZYMATIC DEVELOPMENT OF PYRUVIC ACID AND ODOR IN FROZEN ONIONS TREATED WITH CYSTEINE C-S LYASE. S. SCHWIMMER & D. G. GUADAGNI. *J. Food Sci.* **33,** 193–196 (1968)—Extracts of commercially frozen onion were devoid of strong flavor and of L-cysteine sulfoxide lyase. These extracts were selected as model *in situ* substrates for a study of the kinetics of odor production catalyzed by the L-cysteine C-S lyase of *Albizzia lophanta* seed endosperm. The results suggest that both odor and pyruvic acid may be produced via the same enzyme but that the odor is formed after the formation of pyruvic acid. The odor threshold value of some of the enzymatically produced odor-bearing constituents in onions may be less than one part per billion.

THE FREE AMINO ACIDS OF ISRAEL ORANGE JUICE. B. R. COUSSIN & Z. SAMISH. *J. Food Sci.* **33,** 196–199 (1968)—Twenty-two Israel orange juice samples were analyzed chromatographically, and 16 free amino acids were identified with seven different solvent systems. Aspartic acid, glutamic acid, lysine, alanine, and proline were identified with all seven solvent systems; asparagine with six; serine with five; arginine, valine and leucine with four; γ-amino-butyric acid with three; glycine, methionine and phenylalanine with two; and threonine and tyrosine with one. A quantitative estimation of the free amino acids indicates that amounts of aspartic acid, serine, and alanine are high compared with California orange juice; but glutamic acid and lysine are low.

RIGOR STATE, FREEZE CONDITION, pH, AND INCUBATION TEMPERATURE AND THEIR INFLUENCE ON COLOR DEVELOPMENT AND EXTRACT RELEASE VOLUME IN OVINE MUSCLE HOMOGENATES. C. F. COOK. *J. Food Sci.* **33**, 200–207 (1968)—Buffer-pH, incubation temperature and the interaction between these two factors had a highly significant effect ($P < 0.01$) on filtrate color and ERV. Rigor state and the interactions, rigor state $\times$ buffer-pH, freeze state $\times$ buffer-pH, and freeze state $\times$ incubation temperature had a highly significant effect ($P < 0.01$) upon ERV after 24 hr incubation. Pale colored filtrates developed in homogenates that were buffered at pH 5.2 and 5.6 and incubated at 20, 30, and 40°C.

STEPWISE DISCRIMINANT ANALYSIS OF GAS CHROMATOGRAPHIC DATA AS AN AID IN CLASSIFYING THE FLAVOR QUALITY OF FOODS. J. J. POWERS & E. S. KEITH. *J. Food Sci.* **33**, 207–213 (1968)—By calculating all possible ratios among peak heights and subjecting these ratios to discriminant analysis, coffee could be classified into four flavor categories from the gas chromatographic data. The discriminant analysis procedure was set to select the ratio most critical in differentiating among the chromatograms, then move on to the next most efficient ratio until the samples were classified. The same thing was done for potato chips except headspace volatiles were used for the gas chromatographic analysis. Not only does the procedure described enable flavor to be correlated with gas chromatographic data, but the efficiency values for each ratio are useful.

ORGANOLEPTIC IDENTIFICATION OF ROASTED BEEF, VEAL, LAMB AND PORK AS AFFECTED BY FAT. A. E. WASSERMAN & F. TALLEY. *J. Food Sci.* **33**, 219–223 (1968)—Only about one-third of the panel could identify correctly all four meats by memory of the flavors. Beef and lamb, but not pork and veal, were identified significantly less often when lean ground roasts were tested than when normal ground roasts (containing fat) were used. Texture, color, mouth feel, and other factors may be important in the identification of meat.

DETERMINATION OF FLAVOR THRESHOLD LEVELS AND SUB-THRESHOLD, ADDITIVE, AND CONCENTRATION EFFECTS. E. S. KEITH & J. J. POWERS. *J. Food Sci.* **33**. 213–218 (1968)—The flavor threshold concentrations of 23 compounds composing an artificial peach beverage base were determined. The threshold levels ranged from 52 ppm to 0.4 ppb. Sub-threshold and additive sub-threshold effects resulted from only a few of the flavor combinations tried. Change in concentration of one compound in a mixture of six compounds was not readily detectable organoleptically. The difference in concentration could be detected gas chromatographically, but only if the mixtures were extracted with pentane and concentrated.

TEXTURE PROFILE OF RIPENING PEARS. M. C. BOURNE. *J. Food Sci.* **33,** 224–227 (1968)—Sample of pears in a ripening room were taken out at regular intervals for texture measurements which consisted of the Magness-Taylor pressure test and a modified General Foods Texture Profile. The adhesiveness was zero. The viscosity could not be measured. All other parameters of the G. F. Texture Profile decreased during ripening at approximately the same rate as the pressure test. This characteristic of ripening pears could be responsible for the success of a simple single measurement (the puncture test) for measuring the complex phenomenon "textural quality" of pears.

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Changes in the Polyunsaturated Fatty Acid Content of Potato Tubers During Growth, Maturation and Storage

SUMMARY—Kennebec and Red Pontiac potatoes were analyzed for fatty acids at intervals during growth and maturation of the tubers and subsequent storage at 4°C. During storage linoleic and linolenic were almost the only polyunsaturated acids present, but during growth and maturation considerable amounts of unidentified polyunsaturated acids were found. The percentage of polyunsaturated acids in the dry weight of tuber decreased to a low value near harvest time and remained near this value throughout the 6.3-month storage period, except that the value in Pontiacs stored 19 days was somewhat high. The percent of polyunsaturates in the total fatty acid fraction also dropped to a low value during growth and maturation but increased somewhat during storage. For this reason it may be better, when practicable, to make dehydrated products from freshly-harvested rather than from stored potatoes.

Potato dice contained as much polyunsaturated acid as the tubers they were made from; potato flakes contained somewhat less. In both products the degree of unsaturation of the fatty acid fraction was the same as for the tubers. No off-flavors were noticed when samples were reconstituted. Apparently little or no oxidation took place during the processing.

INTRODUCTION

THE QUANTITY OF LIPIDS in the white potato may be too small to be of nutritional significance. Various authors report values of 0.02 to 0.2 for the percentage of fat on a wet basis, as quoted by Lampitt *et al.* (1940). Völksen (1950) isolated and characterized linoleic and linolenic acids from potato fat but gave no quantitative data. Using spectrophotometric methods, Highlands *et al.* (1954) found 41% linoleic acid and 28% linolenic acid in the fat of Katahdin potatoes. Buttery *et al.* (1961) isolated linoleic and linolenic acids from Russet Burbank potatoes by use of gas chromatography and identified them by their infrared spectra; they reported 53% linoleic acid and 20% linolenic acid.

Because of the highly unsaturated nature of the fatty acids, the small quantity present is responsible for at least part of the oxidative off-flavor formed during the storage of dehydrated potato products. Burton (1949), Hendel *et al.* (1951), and Highlands *et al.* (1954) presented evidence indicating that certain off-flavors in dehydrated potato products are the result of unsaturated fatty acid oxidation. Buttery *et al.* (1961) demonstrated that during the storage of canned potato granules there is a correlation between the decrease in the unsaturation of the fatty acids, the decrease in the oxygen content of the head space, and the increase in off-flavor.

If there is a change in the concentration of polyunsaturated acids in potatoes during maturation or subsequent storage of the tubers, it may be possible to select a time for processing which will favor a reduced tendency toward the development of oxidative rancidity in dehydrated products.

Hilditch (1951) reviewed work showing a notable increase in unsaturation in many oil seeds during the final stage of ripening. For potatoes, little has been published on this subject. Mondy *et al.* (1963) analyzed potatoes stored for 2 to 16 weeks at 4°C. For Red Pontiacs, they found that the mole percentage of linoleic acid decreased from 51 to 7, but the corresponding values for linolenic increased from 16 to 37. However, for the Ontario variety, linoleic remained constant at 28%, and linolenic showed a slight decrease from 37% to 32%. In both varieties, total crude lipids decreased slightly. Cotrufo *et al.* (1964) reported a small increase in total fatty acids (averaged over nine varieties) during two months of storage at 22°C followed by a small decline in subsequent months.

The present study was made to follow changes in the polyunsaturated fatty acid concentrations which occur during growth, maturation, and storage of the tuber and to determine to what extent these acids were oxidized during processing.

EXPERIMENTAL

SAMPLES OF KENNEBEC AND RED PONTIAC potatoes grown in the Red River Valley were dug at intervals during growth and maturation and shipped to this Laboratory by Air Express. Ten pounds of each variety were sent in each of the first four shipments, 25 pounds of each in the fifth shipment. The first shipment, dug August 3, 1965, contained Kennebecs ranging in length from 2.5 to 7.5 cm and Red Pontiacs ranging in length from 3.5 to 7.0 cm. The fourth shipment consisted of potatoes dug on September 15—two days after the vines were sprayed with herbicide. Upon harvest (September 24), samples of the tubers were taken for the fifth shipment; the rest were cured for two weeks at 18°C and then put in storage at 4°C. Samples weighing 25 pounds were subsequently taken at various storage intervals and shipped to this Laboratory.

Upon receipt of the shipments, three representative samples of each variety were obtained by arranging the potatoes in order of size and selecting equal numbers of each size. Although the fat is more concentrated in potato peels than in the rest of the tuber (Völksen, 1950), most of the skin was removed by peeling for 20 sec in an abrasive peeler (Toledo Vegetable Peeler, Model A1-15) to simulate more nearly conditions in the potato processing industries. After this, stem material, buds and

sprouts, deep eyes and bad spots were removed. The potatoes were then rinsed, dried roughly, and French-fry sliced into strips about 6 mm thick. After the strips were mixed, 350-g samples were packed loosely into 125 mm diameter crystallizing dishes. These were placed in polyethylene bags and stored at −20°C until analyzed.

Potato flakes and dice were made by the Red River Valley Potato Processing Laboratory at intervals during storage of the tubers. Flakes were made by the potato flake process developed by Sullivan *et al.* (1961); dice were prepared by cooking diced potatoes and drying the dice in a forced-draft tray dryer. Upon receipt at this Laboratory, the processed products were removed from their bags, sealed in cans under nitrogen, and stored at −20°C.

Freeze-drying

Duplicate samples of the potato strips (the third sample was kept as a reserve) were transferred to stainless-steel screen baskets a little larger in diameter than the crystallizing dishes and freeze-dried on heated metal trays thermostatically controlled at 27°C (Lucite Tray Dryer L-100, Associated Testing Laboratories, Inc.). To follow the course of the drying, small metal thermometers were placed horizontally on the trays. A layer of Wood's metal was melted over the thermometer stems to hold them in place and to insure good heat conduction. Sublimation kept the shelf temperatures low during the freeze-drying; when they had risen to 27°C for a few hours, the drying was stopped.

Preparation of fatty acid methyl esters

The dried potatoes were ground in an Intermediate Model Wiley Mill with a 40-mesh screen. The ground material was mixed in a jar after the head space was filled with nitrogen, and duplicate 1 g samples were taken for determining solids by oven-drying at 110°C.

The potato lipids were saponified and the fatty acids extracted by a modification of the method used by Buttery *et al.* (1961) for potato granules. Sixty-g samples were mixed with a solution of 28.0 g KOH (assay 86%) in 350 ml of 90% ethanol. At this point, approximately 18 mg of methyl nonadecanoate or 30 mg of methyl behenate, weighed accurately on a microbalance, was added as a standard. The mixture was allowed to stand four days at room temperature and then filtered. The filtrate was concentrated in a rotary evaporator with bath temperatures below 38°C. After extraction of the unsaponifiables, the mixture was acidified to pH 2.0 with concentrated HCl (pH meter) to obtain the free acids.

Methyl esters were prepared by refluxing for 75 min a mixture of the acids in 5 ml of hexane with 70 ml of 5% H_2SO_4 (w/v) in dry methanol containing a crystal of hydroquinone. The reaction mixture was cooled and 30 ml of water was added. The mixture was then extracted with hexane and the resulting extract was washed with water and dried overnight with 4:1 anhydrous Na_2SO_4-$NaHCO_3$. It was then concentrated to about 5 ml under a nitrogen jet, passed through a 1.3 cm long 1.0 cm diameter column of Florisil, and concentrated further to about ¼ ml in a sample tube which had been drawn to a point.

Gas chromatography

Samples were analyzed on an F & M Model 810 Chromatograph with a thermal conductivity detector. The columns used were 8-ft by ¼-in. stainless steel tubing packed with 10% diethylene glycol succinate (Applied Science Laboratories, Pretested Grade) on 70–80 mesh Gas-Chrom RZ. Runs were made isothermally at 218°C with a helium flow of 60 ml/min. An Infotronics Model CRS-11HS digital integrator, connected directly to the output of the detector cell, was used to measure relative peak areas.

Methyl nonadecanoate was used as standard for the analysis of the first four potato shipments. However, by the fifth shipment it was apparent that only a slight deterioration of the chromatographic column was resulting in poor resolution of the nonadecanoate and linolenate peaks. For this reason both nonadecanoate and behenate were used for analysis of the fifth shipment, and only behenate was used in subsequent analyses, because its elution time was sufficiently different from that of all the potato acids. No difference was found between results based on the two standards.

RESULTS AND DISCUSSION

Two types of determination have a bearing on this study—the percent of the total potato solids represented by the polyunsaturated acids and the percent of the total fatty acid fraction represented by the polyunsaturated acids. The concentration of these acids in the total solids should affect the amount of oxidation products that accumulate in dehydrated potatoes. This value was calculated by comparing peak areas with the peak area produced by a known quantity of standard. The concentration of polyunsaturates in the total fatty acids, which was calculated from relative peak areas alone, should affect the rate at which the oxidation products form (Bolland, 1948; Buttery *et al.*, 1961).

To calibrate the gas chromatographic method, weighed amounts of methyl behenate and a standard mixture of methyl palmitate, stearate, oleate, linoleate and linolenate (Applied Science Laboratories, Inc. mixture K-108) were made up to volume in separate flasks. The two solutions were mixed in varying proportions and samples were run on the gas chromatograph. Ratios of the weight of each ester to that of the methyl behenate ranged from 0.2 to 3.6. Sample sizes were chosen to give a methyl behenate peak area similar to that which was maintained in analyzing the potato samples. Plots of weight ratios of each ester to behenate versus the corresponding peak area ratios showed a linear relationship. Although detector response was somewhat lower for late-appearing peaks, correction factors were not used because errors were too small to affect conclusions concerning change with tuber maturity or storage time.

The over-all precision of the analytical method was estimated for linoleic and linolenic acids by calculating the coefficient of variation (the Standard Error for duplicate determinations × 100 divided by the mean). This value differed somewhat depending on whether the seven larger values for each ester (seven including results for both varieties, Tables 1 and 2) or the 10 smaller values

Table 1. Changes in acid concentrations in Red Pontiac potatoes.[1]

	Maturation					Storage			
Date	8/3/65	8/16	8/30	9/15	9/24[2]	10/13	11/23	1/24/66	3/30
Palmitic	.27	.29	.34	.12	.12	.19	.094	.098	.095
Stearic	.026	.029	.026	.017	.020	.042	.015	.016	.016
Oleic	.010	.012	.015	.004	.005	.009	.003	.003	.009
Linoleic	.46	.64	.50	.094	.14	.29	.17	.16	.14
Linolenic	.24	.33	.29	.044	.062	.14	.074	.088	.10
Unidentified[3]	.14	.051	.33	.059	.026	.002	.001	0	0
Total acids	1.21	1.42	1.63	.35	.38	.72	.36	.37	.37
Percent unsaturates[4]	73	75	74	58	60	64	68	67	66

[1] Average of duplicate determinations reported as percent methyl ester on potato dry matter.
[2] Date of harvest; potatoes were planted on 5/28.
[3] Includes only one peak; other unidentified peaks very small.
[4] Percent polyunsaturated esters based on total fatty acid esters.

Table 2. Changes in acid concentrations in Kennebec potatoes.[1]

	Maturation				Storage			
Date	8/3/65	8/16	8/30	9/15[2]	10/13	11/22	1/24/66	3/29
Palmitic	.19	.31	.22	.11	.12	.084	.079	.078
Stearic	.024	.029	.023	.020	.022	.017	.015	.017
Oleic	.007	.014	.009	.006	.005	.003	.003	.004
Linoleic	.47	.48	.36	.18	.17	.16	.17	.14
Linolenic	.16	.19	.14	.064	.054	.071	.076	.074
Unidentified[3]	.....	.32	.15	.027	.026	.004	0	0
Total acids	.88	1.48	.95	.42	.41	.35	.35	.32
Percent unsaturates[4]	73	73	72	66	62	68	71	68

[1] Average of duplicate determinations reported as percent methyl ester on potato dry matter.
[2] Vines killed on 9/13; potatoes were planted on 5/28, harvested on 9/24.
[3] Includes only one peak; other unidentified peaks very small.
[4] Percent polyunsaturated esters based on total fatty acid esters.

were used in its calculation. The coefficients for the linoleate determination were 11.3% for the large values and 8.1% for the small; the corresponding coefficients for the linolenate determination were 13.2% and 12.9%.

Changes in the concentrations of linoleic and linolenic acids in the tubers both followed the same trend. Fig. 1 shows changes in the sum of the two and also changes in concentration of the largest unidentified peak. Mass spectroscopy indicated the latter to be a mixture with molecular weights corresponding to those of long-chain polyunsaturated acids. Although not fully identified, the mixture does not affect the overall conclusions since its concentration mainly follows the same trend as that of the known polyunsaturated acids. Other unidentified peaks were too small to be of consequence.

Fig. 1 shows that in immature tubers the percent of polyunsaturates on the dry weight of potatoes is very high, falls to a low point near harvest time, and then remains fairly constant during storage. Exceptional is the high value in Pontiacs which had been stored 19 days. Results calculated on a fresh-weight basis would be similar for the stored potatoes since the concentration of solids does not change during storage. However, since immature tubers are low in solids (Murphy *et al.*, 1959), fresh-basis values would be lower for this period.

In Fig. 2 the percent of polyunsaturated acids in the total fatty acids is shown. As in the case of the percent based on total solids, this value for the Pontiacs starts out high in the immature potatoes and soon falls to a low point near harvest time. For the Kennebecs the trend is similar, but no data were obtained on the freshly-harvested potatoes; the low point shown is for tubers stored 19 days. In both varieties the fatty acid fraction of stored potatoes is

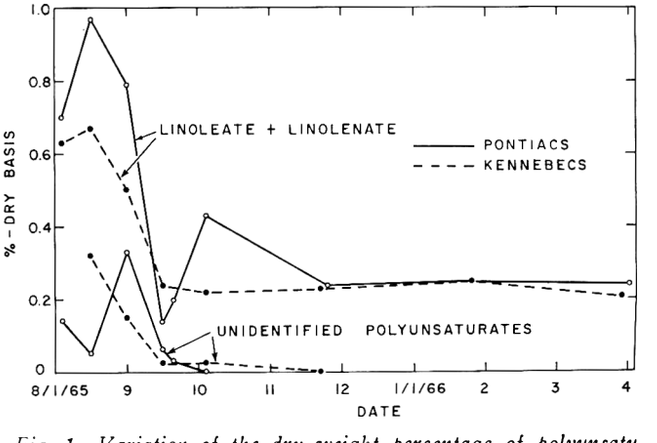

Fig. 1. Variation of the dry weight percentage of polyunsaturated fatty acids in tubers with growth, maturation, and storage time. The potatoes were harvested on 9/24/65. Results are plotted as percent methyl ester on dry weight of tuber.

Table 3. Acid concentrations in flakes prepared from potatoes stored for various periods.[1]

Date of preparation	Red Pontiacs				Kennebecs			
	10/8/65	11/16	1/20/66	3/29	10/8/65	11/16	1/20/66	3/29
Palmitic	.....[2]	.....	.053	.077	.....[2]	.....	.047	.054
Stearic	.....	.....	.011	.013	.....	.....	.011	.011
Oleic	.....	.....	.002	.002	.....	.....	.002	.001
Linoleic	.15	.19	.10	.13	.14	.13	.11	.11
Linolenic	.051	.065	.039	.074	.040	.044	.039	.051
Total acids	.....	.....	.21	.30	.....	.....	.21	.23
Percent unsaturates[3]	.....	.....	66	68	.....	.....	70	70

[1] Average of duplicate determinations reported as percent methyl ester on potato dry matter.
[2] The first two samples of flakes (of each variety) had been treated with an additive.
[3] Percent polyunsaturated esters based on total fatty acid esters.

more unsaturated than that of potatoes analyzed near harvest time.

From both Figures it appears that nothing can be gained by processing somewhat immature potatoes but, from the standpoint of the degree of unsaturation of the fatty acid fraction, it is better to process freshly-harvested potatoes than stored potatoes. However, this is not often practical since most dehydration plants must use stored potatoes.

The concentrations of palmitic, stearic, and oleic acids in the potato solids, and that of the total acids (Tables 1 and 2), follow the same trends noted for the polyunsaturated acids during the growth and maturation of the tuber.

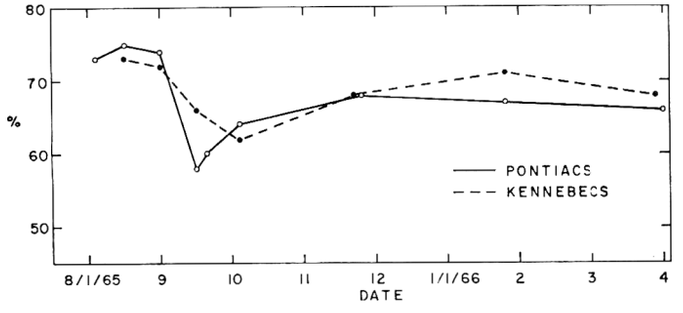

Fig. 2. Variation of relative concentrations of polyunsaturated acids in the fatty acid fraction during growth, maturation, and storage of tubers. The potatoes were harvested on 9/24/65. Results are plotted as percent methyl ester on total weight of fatty acid esters.

Table 4. Acid concentrations in dice prepared from potatoes stored for various periods.[1]

Date of preparation	Red Pontiacs			Kennebecs		
	11/24/65	1/20/66	3/28	11/24/65	1/20/66	3/28
Palmitic	.069	.090	.093	.089	.074	.073
Stearic	.014	.013	.014	.014	.015	.015
Oleic	.004	.004	.004	.003	.004	.003
Linoleic	.16	.16	.17	.17	.16	.14
Linolenic	.053	.074	.10	.061	.067	.071
Total acids	.31	.35	.39	.35	.32	.31
Percent unsaturates[2]	70	68	70	68	70	69

[1] Average of duplicate determinations reported as percent methyl ester on dry matter.
[2] Percent polyunsaturated esters based on total fatty acid esters.

As in the case of the latter acids, changes during growth and maturation were much more pronounced than during storage.

Fatty acid composition of the potato flakes and dice is shown in Tables 3 and 4. The dice contain about the same amount of unsaturates as the tubers from which they were made; the flakes contain somewhat less. In both products the degree of unsaturation of the fatty acid fraction is the same as for the tubers. No off-flavors were noticed when samples of the flakes and dice were reconstituted. Apparently little or no oxidation took place during the processing.

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Mention of company or product names does not imply recommendation of the products by the U. S. Department of Agriculture over others that may be suitable.

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Identifying Nucleotidic Materials Released by Fermenting Brewer's Yeast

SUMMARY—During fermentation a brewer's yeast released into the medium substantial amounts of materials absorbing ultra-violet light at 260 mμ. These materials were separated by chromatography and identified by further chromatography and spectrophotometry. Nine nucleotides, five free bases (purines and pyrimidines) and four nucleosides were identified in the fermented medium and in the intracellular pool of the yeast. Some of these released materials may be of consequence in the fermented beverage industry since they were not reabsorbed by yeast and can be expected to persist to the finished product.

INTRODUCTION

IT HAS BEEN KNOWN for many years that when yeast fermented a sugar solution, nitrogen-containing substances were released into the medium. Some of the released substances were amino acids and some strongly absorbed U.V.-light at 260 mμ. Delisle *et al.* (1961) showed that the U.V.-absorbing fraction was nucleotidic material of low molecular weight which was released from viable healthy yeast cells; *i.e.,* leakage was not the result of autolysis. Higuchi *et al.* (1959) considered leakage of nucleotides to be the result of enzymic degradation of RNA.

Lewis *et al.* (1963) showed that nucleotide release was stimulated by conditions favoring fermentation and growth, and that the released material was not subsequently utilized by the yeast. It seemer plain, therefore, that in fermentation to produce beer or wine, the released material would contribute to the composition of the finished product.

This work was undertaken to determine the nature of the material released under simple conditions of fermentation in an attempt to evaluate the importance of the release of nucleotidic material in beer or wine quality.

METHODS AND MATERIALS

Test organism

The yeast used (yeast L) was a flocculent commercial strain of *Saccharomyces carlsbergensis.* It was grown as previously described (Lewis *et al.,* 1964) under anaerobic conditions in a medium rich in organic nitrogen containing glucose as fermentable sugar. The yeast was grown for three days, harvested by centrifugation, washed three times in distilled water and used immediately.

Fermentation experiments were conducted in a 2% w/v solution of glucose for 3 to 5 hr at room temperature using the yeast at a concentration of 10 mg (dry wt) per ml. At the end of this period the glucose was exhausted.

The material in this paper is part of a thesis submitted by T. C. Lee to the Graduate School of the University of California in partial satisfaction of the requirements for the M.S. degree in Food Science.

Concentration of nucleotidic material

The yeast was removed from the fermented medium by centrifugation and filtration (Millipore filter). The nucleotidic fraction was then concentrated by Nakajima's (1963) method: the fermented medium was adjusted to pH 2.0 with 1.0 *N* HCl and passed through a column of activated charcoal. The column was thoroughly washed with distilled water to remove interfering substances. Nucleotidic materials were then eluted from the column with 15 ml of 3.0% ammonia in 50% ethyl alcohol at a flow rate of 0.5 ml/min. The eluate was evaporated to dryness and the residue was redissolved in a small amount of distilled water. Recovery of nucleotides by this method was about 95%.

Intracellular nucleotides were extracted by exposing the yeast to boiling water for 20 min.

Chromatography

The sample was adjusted to pH 6 to 7 and applied to a column of Dowex-1 purchased in the formate form (AG-1-X8, 200 to 400 mesh Bio-Rad Laboratories, Richmond, Calif.). The column was 23 cm high and 10 mm in diameter. After the sample was applied, the column was washed with distilled water until the O.D. 260 of the eluate was zero. This procedure removed nucleosides and purine and pyridine bases from the column. The nucleotides were then eluted using a formic acid/formate gradient.

A four-bottle system was used as shown in Fig. 1. The bottles contained (1) 500 ml distilled water, (2) 500 ml distilled water, (3) 500 ml of 0.5 *N* formic acid and (4) 500 ml of 0.5 *N* formic acid containing 0.5 *M* ammonium formate. The gradient took about 40 hr for completion. The column was finally flushed with 200 ml of 4 *N* formic acid containing 0.8 *M* ammonium formate. The fractions corresponding to each peak were pooled and the O.D. 260 of each separated peak was used to calculate the amount of nucleotide present.

The material was then concentrated in a rotary evaporator and treated by Nakajima's (1963) method to remove formic acid and formate. The samples were then ready for further chromatography on a column (where separation was inadequate) thin layer plates or on paper. The nucleoside/free base fraction was examined directly by paper or thin-layer chromatography.

For paper chromatography Whatman No. 1 paper was used. The two solvent systems chosen were:

1. Isobutanol : water : conc. NH_4OH (66 : 33 : 1)
2. n-Butanol : water : acetic acid (4 : 1.7 : 1)

The chromatograms were developed for 17 to 20 hr by conventional descending-front techniques. The paper was then dried in air and the U.V absorbing materials were located on the paper with a short wave-length ultraviolet

lamp (maximum emission 250 μ, Mineral Light, Ultra Violet Products Inc., San Gabriel, Calif.). Comparison was made to standards.

For thin-layer chromatography, plates (20 × 20 cm) were prepared according to Stahl (1965) using MN-300 cellulose powder (Macherey, Nagel and Co., Düren/Khld., Germany). Chromatograms were developed in tert-amyl alcohol:formic acid:water (3:2:1) or in saturated ammonium sulfate:1 *M* sodium citrate:isopropanol (40:9:1) by ascending front for 3 hr. The nucleoside/free base fraction was also determined using distilled water as developing agent. Materials were located as before using the short wave-length lamp. Comparison was made to standards.

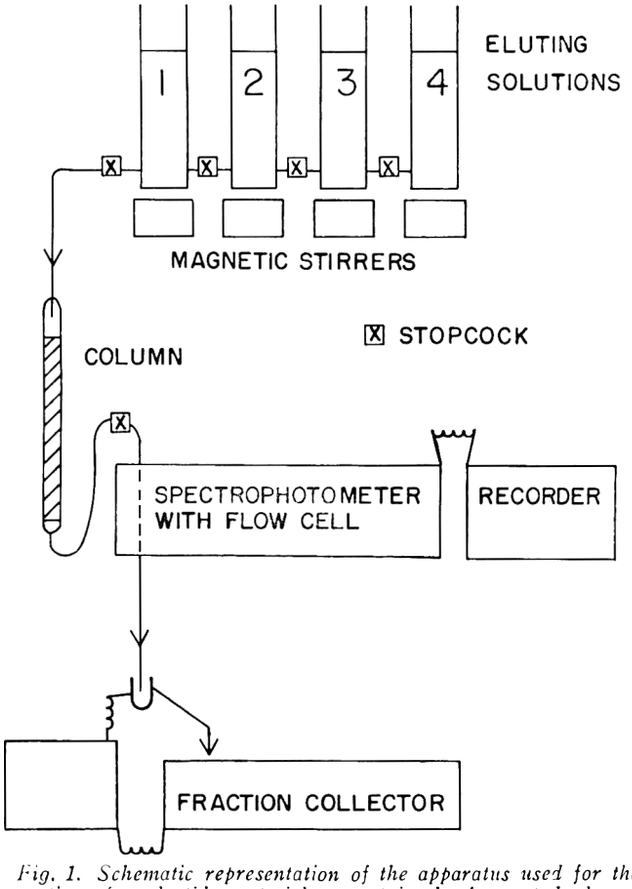

Fig. 1. Schematic representation of the apparatus used for the separation of nucleotide material present in the fermented glucose solution or extracted from yeast.

Spectrophotometric analysis

After chromatographic separation, suitably diluted samples were examined spectrophotometrically at acid (pH 2) neutral (pH 7) and alkaline (pH 11) pH. These pH levels were established with 0.01 *N* HCl, 0.02 *M* phosphate buffer and 0.002 *N* NaOH respectively. The spectra were determined in a Beckman DK recording spectrophotometer and compared to authentic standards.

Fractionation of beer

The nucleotidic material in degassed beer was concentrated by Nakajima's (1963) method described above. The material eluted from the charcoal column was further fractionated into nucleotide or nucleoside/free base fractions by its affinity or lack of affinity for a column of Dowex-1 formate-form.

RESULTS

Characteristics of the release material

By treatment with Dowex-1 resin, nucleotidic material was readily divided into two fractions: a nucleotide fraction and a nucleoside/free base fraction. The material released into the medium and the material extracted from the yeast were separated in this way.

The results of this fractionation are shown in Table 1. It can be seen that during fermentation, there was a tendency for the intracellular nucleoside fraction to increase somewhat at the expense of the nucleotide fraction (compare yeast in glucose solution to yeast in water). Similarly the material released from yeast suspended in water or glucose solution contained a rather higher proportion of nucleosides than did the intracellular material. These observations could be explained by selective utilization of nucleotides during fermentation, or by dephosphorylation

Table 1. Relationship of nucleosides and free bases (B) or nucleotides (C) to ultraviolet absorbing substances (A), (a) in the pool, or (b) released into the medium.

Sample	(A) Total O.D. at 260 mμ	(B) Nucleosides and free bases. O.D. at 260 mμ	(C) Nucleotides O.D. at 260 mμ	Nucleosides as % of total (B)/(A) × 100	Nucleotides as % of total (C)/(A) × 100	Nucleotide triphosphates[1]
Extracellular level (b) after exposure of yeast to glucose solution	850	355	495	41%	59%	absent
Intracellular level (a) after exposure of yeast to glucose solution	2830	765	2065	25%	75%	present
Extracellular level (b) after exposure of yeast to water	170	37	133	18%	82%	absent
Intracellular level (a) after exposure of yeast to water	3150	334	2816	11%	89%	present

[1] Material remaining on Dowex-1 column after completion of gradient elution.

of nucleotides during fermentation or release. The material released during fermentation was found to be 60% nucleotide and 40% nucleoside/free base.

Identification of Nucleotides

A concentrated sample of nucleotidic material obtained from the fermented medium or extracted from the yeast was applied to a Dowex-1 column (formate form) as described. The column was washed with distilled water until the eluate had an optical density of zero at 260 mμ. The formic acid/formate elution gradient was then begun. The position of elution of the peak from the column was a preliminary indication of identity. Fractions containing the individual peaks were pooled, concentrated, and then applied to thin layer plates and paper chromatograms. A number of standards were included on each chromatogram to provide identification. Identity was considered proven if a peak moved on thin layer plates and on paper in several solvent systems with one authentic standard. In most cases this was further confirmed by examining the U.V.-absorption spectrum of the unknown at several pH values and comparing this to the standard.

The trace shown in Fig. 2 was rather routinely obtained from chromatographic analysis of the nucleotide fraction of the medium on an ion exchange column. The identities of the peaks are given. Fig. 3 shows a chromatographic separation of the U.V.-absorbing material extracted from the yeast. The intracellular material was found to be qualitatively similar in composition to that of the released material (Fig. 2), and no compound was identified in the medium that was not also present intracellularly. Release of nucleotides by yeast suspended in water is shown in Fig. 4.

Table 2 shows the amount of individual nucleotides released by yeast or extracted from the intracellular pool. As indicated in Table 1, the released material was related qualitatively but not quantitatively to the material present in the intracellular pool.

In Fig. 2, 3, 4 and Table 2, the location of the phosphate group on the ribose ring is given as being at the 5′-position. This was demonstrated by using a specific enzyme method described by Nakajima (1963). The individual peaks were collected and concentrated and reacted with the enzyme 5′-nucleotidase (Takeda Chemical Industries, Park Avenue, N. Y.). The release of inorganic phosphate was

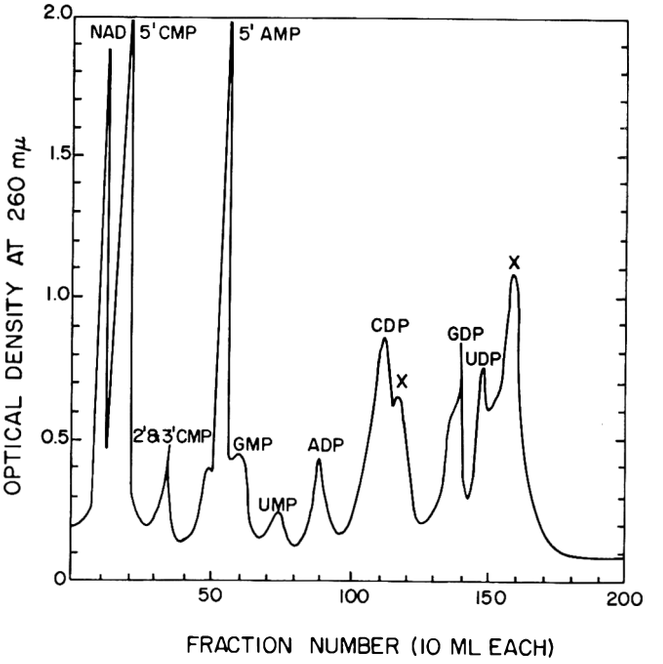

Fig. 3. Separation of nucleotidic material extracted from yeast L after fermentation for 3 hr in a solution of glucose.

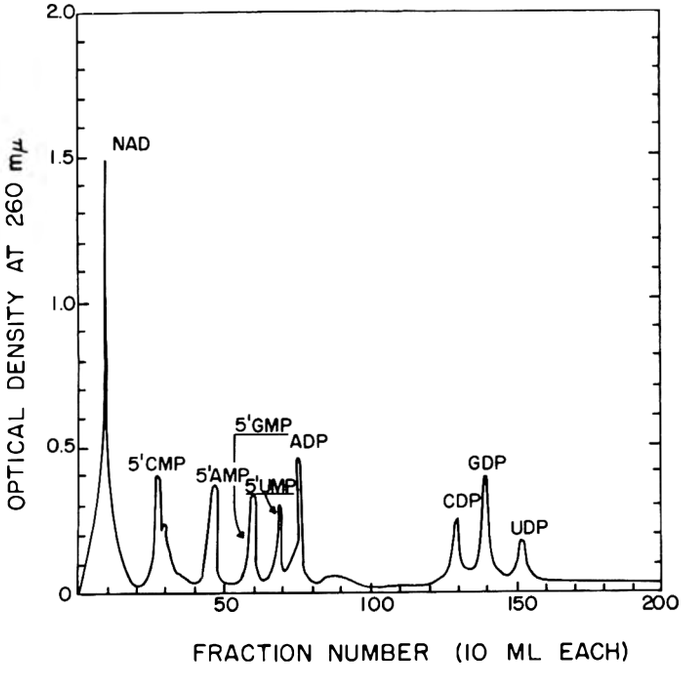

Fig. 2. Separation of nucleotides released by yeast L during fermentation of glucose solution for 3 hr.

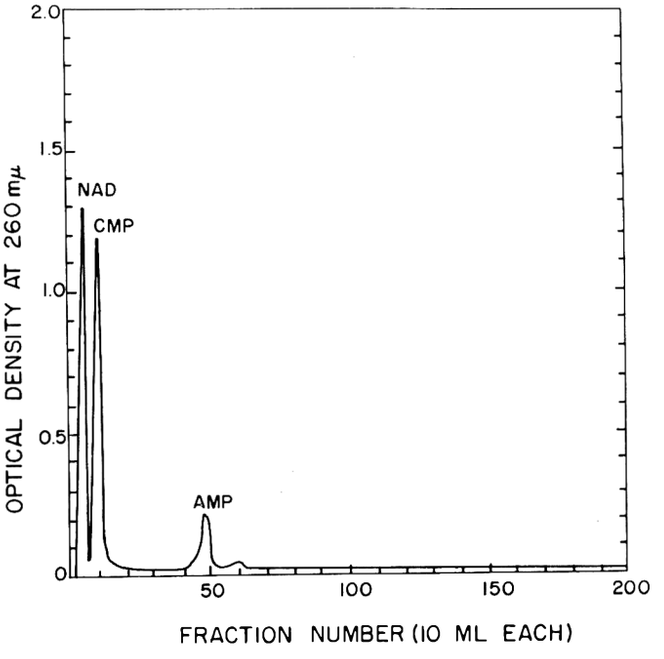

Fig. 4. Separation of nucleotides released by yeast L suspended for 3 hr in distilled water.

Table 2. Nucleotides released by yeast or present in the intracellular pool after 3 hr of incubation.[1]

	Yeast suspended in water:—		Yeast suspended in glucose solution:—	
	Extracellular	Intracellular	Extracellular	Intracellular
Nicotinamide-adenine dinucleotide	70	660	163	330
cytidine 5′-monophosphate	70	370	69	645
adenosine 5′-monophosphate	32	600	57	690
guanosine 5′-monophosphate	—	430	47	167
uridine 5′-monophosphate	—	290	47	143
adenosine-diphosphate	—	192	63	194
cytidine-diphosphate	—	860	63	575
guanosine-diphosphate	—	475	77	370
uridine-diphosphate	—	1010	33	320
Triphosphate or oligo-nucleotides	—	+	—	+

[1] Nucleotides are reported as μg/g of yeast, dry weight. — absent, + present.

taken as proof of a phosphate group at the 5′-position since the enzyme was found to be inactive on phosphate groups located at the 2′- or 3′-position of the ribose ring.

The identity of nicotinamide-adenine dinuclotide (NAD) was confirmed using alcohol dehydrogenase (Worthington Biochemical Corp., Freehold, N. J.), with ethanol as substrate. When these reagents were mixed with the appropriate peak eluted from the column, there was a rise in optical density at 340 mμ.

Nucleosides and free bases

Those materials which had no affinity for the Dowex-1 column, nucleosides and pyrimidine and purine bases, were examined using thin layer plates and paper chromatography in several solvent systems. If an unknown peak moved in all cases with a known standard, it was considered identical to that standard. The materials so identified are shown in Table 3. Hypoxanthine was one of the bases identified.

Nucleotidic material of beer

Beer has a very high optical density at 260 mμ. When a simple fractionation procedure was applied, it was found that 42% of the U.V.-absorbing substances of beer was non-nucleotidic. Of the 58% nucleotidic material, 62% was nucleotide, 32% was nucleoside/free base and 6% was nucleotide triphosphate and oligo-nucleotides. These materials were identified by the usual procedures and are shown in Table 4.

The nucleotidic material of beer could originate in the malt or hops as well as yeast. The rather limited range of materials found in beer was somewhat surprising. It seems possible, however, that much of the nucleotidic material derived from malt or hops may be lost in the mashing and boiling processes, possibly as co-precipitates with protein. Under such conditions the bulk of the nucleotidic fraction of beer may be derived from yeast.

Table 3. Nucleosides and free bases released by yeast during fermentation of glucose solution at 25°C, or extracted from the yeast.

Free bases:	
	Adenine
	Cytosine
	Guanine
	Uracil
	Hypoxanthine
Nucleosides:	
	Adenosine
	Cytidine
	Uridine
	Guanosine

DISCUSSION

In brewery fermentations the character of the finished beer is in large measure determined by the yeast used. This is especially noticeable in the low-molecular weight volatile components of the product.

Challinor *et al.* (1954) demonstrated that a fastidious strain of lactobacillus was able to grow on a simple medium previously fermented by yeast. Nicotinic acid or its amide was one of the components released by yeast and utilized by the lactobacillus. Kulka (1963) showed that the biological stability of British ales was related to the capacity of the yeast to autolyze or leak. These observations support the suggestion made here that the nucleotidic fraction of beer may originate primarily in the yeast since, if a large proportion was derived from malt, the effect of yeast strain on biological stability would not be appreciable. Those nucleotidic materials released by yeast were also found in beer.

Reich *et al.* (1958) attempted to analyze all the nitrogen-containing substances in beer. They concluded in part that beer contains nucleotide 5′-monophosphates. This

Table 4. Nucleotidic materials identified in commercial beer.

Free bases:	uracil	
	adenine	
	guanine	
	hypoxanthine	
Nucleosides:	adenosine	
	uridine	
	guanosine	
Nucleotides:	adenosine	5′-monophosphate
	cytidine	5′-monophosphate
	guanosine	5′-monophosphate
Other:	Nicotinamide-adenine dinucleotide and its reduced form, nucleotide triphosphate, and oligo-nucleotides.	

conclusion is amply supported by this work. The bulk of the nucleotides released by this strain of yeast were 5′-monophosphates which appeared to originate in an intracellular free pool of nucleotides. The composition of the intracellular free pool and the released material was qualitatively similar. Nucleotides are synthesized as 5′-monophosphates. It was suggested, therefore, that these materials are released prior to their further synthesis into the ribonucleic acid. The degradation of RNA by RNase would be expected to lead to the production of 2′- and 3′-monophosphates and to oligo- and poly-nucleotides as demonstrated by Higuchi *et al.* (1949) for *S. cerevisiae*. Such products were not found under our conditions.

Inosine 5′-monophosphate and guanosine 5′-monophosphate have been shown to have marked flavor-enhancing properties (Kuninaka *et al.*, 1964). Guanosine 5′-monophosphate was released by yeast in small quantities and was identified in beer. The effectiveness of these flavor enhancers depends on the nature of the food in which they are present. It is, therefore, difficult to say whether the amount released by yeast and present in beer is sufficient to affect beer flavor.

Spinelli (1965) showed that hypoxanthine has an intensely bitter and dry flavor in aqueous solution. The predominant quality of beer flavor is bitterness, normally thought to be derived from hop resins. It is possible, however, that hypoxanthine found to be released by yeast during fermentation can contribute to this flavor sensation of beer. The following sequence of enzymatic reactions has been found in fish meat during storage. As the meat lost freshness IMP was converted to hypoxanthine (Saito, 1960).

$$\text{ATP} \rightarrow \text{ADP} \rightarrow \text{AMP} \rightarrow \text{IMP} \rightarrow \text{INOSINE} \rightarrow \text{hypoxanthine}$$

It seems possible that a related series of changes may occur in beer during fermentation and especially in storage in the presence of yeast (lagering). Such a reaction could be associated with the important flavor changes which occur during the maturation of beer.

The presence of NAD in the fermented glucose medium and beer has been shown. This material clearly leaked from the cytoplasmic content (the pool) of the yeast. In beer this material could contribute to the redox systems present and possibly participate in oxidation-reduction reactions under certain conditions.

Little attention has been given to the leakage of nucleotidic material by yeast as one of the variables in manufacture of beer or wine. This leakage does vary somewhat from yeast to yeast (Lewis *et al.*, 1964). There is good reason to believe however, that yeast can make a substantial contribution to alcoholic beverages through the leakage of nucleotidic material (Lewis, 1964). These leakage products may be of consequence in flavor, physical stability and biological stability of the beverage.

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Mechanism of Release of Nucleotidic Material by Fermenting Brewer's Yeast

SUMMARY—When a strain of brewer's yeast grown under suitable conditions was suspended in a solution of fermentable sugar, nucleotides and other U.V.-absorbing materials were rapidly released from the cells. The extent of release was dependent on the pH of the medium, the temperature, the concentration of fermentable sugar and on the presence of membrane-protecting (Ca^{++} or Mg^{++}) or membrane-damaging (butanol, detergent) reagents. The released material was of low molecular weight and appeared to originate in a free intracellular pool. It was concluded that the mechanism of release of nucleotidic material was the result of a change in permeability of the cytoplasmic membrane of yeast contingent upon the transport and metabolism of fermentable sugar. Leakage of nucleotidic material from yeast was considered to be a normal physiological process of consequence in the brewing and wine-making industries.

INTRODUCTION

FOR MANY YEARS it has been known that during fermentation yeast can release material containing nitrogen into the medium. Delisle *et al.* (1961) and Lewis (1964) have reviewed the literature.

The released material consisted mainly of amino acids and related substances, and U.V.-absorbing compounds. The release of amino acids by yeast was studied in some detail by Lewis *et al.* (1964, 1965) and a mechanism for the release was proposed by Lewis *et al.* (1967). These workers showed that amino acids were released from an intracellular pool during a temporary lag in the absorption of amino acids by this yeast, and during a time (fermentation) when the cytoplasmic membrane was temporarily rendered more permeable. It appeared that the same mechanism was responsible for the release of U.V.-absorbing (nucleotidic) materials. The appearance of U.V.-absorbing compounds in the medium is, therefore, considered to be the result of simple leakage through the cytoplasmic membrane of the yeast and that this is a normal physiological phenomenon. Leakage is markedly increased by fermentation, and data are presented to show that fermentation can increase the permeability of the cytoplasmic membrane.

It is suggested that this may be of consequence in the production of beer or wine where the leaked material may affect the quality of the beverage.

MATERIALS AND METHODS

THE YEAST USED (yeast L) was the same flocculent commercial strain of *S. carlsbergensis* as was used in the work presented in Lee *et al.* (1968). It was grown and harvested in the same way.

Tests for the release of nucleotides during fermentation were conducted in 60-ml volumes of distilled water in 125-ml Erlenmeyer flasks incubated at room temperature (24°C). Glucose concentration was 10% w/v, and the concentration of yeast used was 10 mg (dry wt) per ml, except where otherwise stated.

Samples were taken at intervals and centrifuged twice to remove yeast. Optical density of the fermented liquid was determined in a Beckman DB Spectrophotometer at 260 mμ. The usual experimental period was 3 hr. Intracellular nucleotidic material was extracted by exposing the yeast from a sample of known volume to boiling water for 20 min.

Calcium and magnesium ions were used as the chloride salt (analytical grade reagent). Normal-butanol (Eastman Chemical Co., Rochester, N. Y.) was well shaken with the aqueous solution to achieve solution before addition of the yeast.

RESULTS

Nature of released material

Higuchi *et al.* (1959) showed that a substantial proportion of the U.V.-absorbing materials released by a saki yeast (*S. cerevisiae*) were oligo- and poly-nucleotides. This observation suggested autolysis, although the work was performed at 30°C. Joslyn *et al.* (1955) showed that exposure to higher temperature than this was usually necessary for yeast autolysis and subsequent release of higher molecular weight materials.

To demonstrate the absence of autolysis under our conditions the released material was tested in several ways as shown in Table 1. It was found that the released material was freely dialysable; no material was precipitated by 12.5% trichloro acetic acid, indicating the absence of proteinaceous matter. No precipitate was formed in 10% sodium chloride solution, indicating the absence of high molecular weight nucleic acid material. Burton's (1956) test was negative showing the absence of deoxy-sugars which could have been derived from DNA during autolysis. As expected, the orcinol test (Umbreit *et al.*, 1957) for ribose was positive. It was concluded that the materials released by yeast during fermentation were not derived

Table 1. Characteristics of the released material.

Test	Result	Conclusion
10% NaCl	No precipitate	No nucleic acid
12.5% TCA [1]	No precipitate	No protein
Orcinol test	Positive	Ribose present
Burton's test	Negative	Deoxy ribose absent
Dialysis	Wholly dialysable	Small molecular weight

[1] TCA—trichloroacetic acid.

Table 2. Relationship between the nucleotidic material released into the medium and that of the intracellular pool.

Treatment	Source of sample	Optical density at 260 mμ			
		0 hr	1 hr	2 hr	3 hr
10% Glucose	1. medium	0.45	1.6	2.0	2.3
	2. yeast	13.20	11.8	11.0	10.5
	Total 1 + 2	13.65	13.4	13.0	12.8
Water	1. medium	0.2	0.28	0.32	0.40
	2. yeast	13.4	13.4	13.3	13.4
	Total 1 + 2	13.6	13.68	13.62	13.8

from autolysis. Delisle *et al.* (1961a) reached a similar conclusion.

Origin of the released material

The yeast cell contains a free pool of nucleotidic material analogous to the free pool of amino acids (Spiegelman *et al.*, 1955) situated close to the cell wall. This free pool is a possible source of the released material. If this is so, then some relationships should exist between the free pool and the released material.

Fig. 1 shows the effect of the size of the intracellular nucleotide pool on release of U.V.-absorbing substances. It can be clearly seen that with a smaller pool, less material was released into the suspending medium. Such a relationship was also demonstrated for amino acids (Lewis *et al.*, 1964).

If the free pool of nucleotides is the source of the released material, then the release of some nucleotidic material into the medium should result in a decrease in the intracellular pool. This proved to be the case as shown in Table 2. The total amount of extractable nucleotidic material in the system decreased slightly through the experimental period when yeast was suspended in a glucose solution. If the released material originated from the hydrolysis of RNA (Higuchi *et al.*, 1959) then the total extractable nucleotides of the system would be expected to increase. In addition, the action of ribonuclease on RNA yields nucleotide 3′-monophosphates, which were not detected in the released material (Lee *et al.*, 1968). The released nucleotides were 5′-monophosphates, which is the configuration of nucleotides during their synthesis and prior to their condensation into nucleic acids.

The data contained in the preceding paper clearly showed that those U.V.-absorbing materials present in the internal pool also appeared in the medium during fermentation. No compound appeared extracellularly that was not also present intracellularly. This observation supports the free pool as the origin of the released material. The positive identification of nicotinamide-adenine dinucleotide (NAD) and hypoxanthine in the medium is also clear indication that RNA is not the source of the released material.

If the release of nucleotidic material is a phenomenon of leakage from an intracellular pool, as with amino acids, then some parameters already defined for amino acid leakage should also apply here. Lewis *et al.* (1964) showed that fermentable sugar was a principal requirement for leakage of amino acids to occur. This was found true for nucleotide leakage also (Table 2); i.e., leakage was a glucose-stimulated event. At low levels of sugar, amino acid leakage was linear with sugar concentration. As shown in Fig. 2 this was found to be true also for nucleotide leakage. Leakage of nucleotides was linear up to 0.6% glucose and reached a maximum at about 1.8% glucose. As with release of amino acids, nucleotide leakage was found to be temperature-dependent (Fig. 3). A marked depression of leakage was found below 20°C.

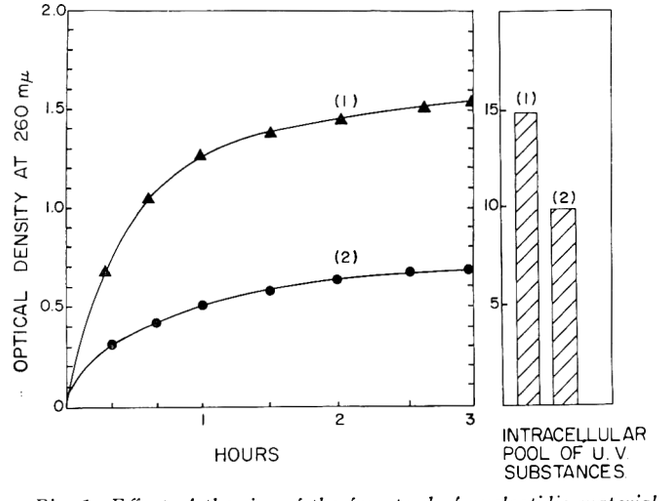

Fig. 1. Effect of the size of the free pool of nucleotidic material on the release of these materials during fermentation. The size of the free pool of yeast L was reduced by growing the yeast in shake culture.

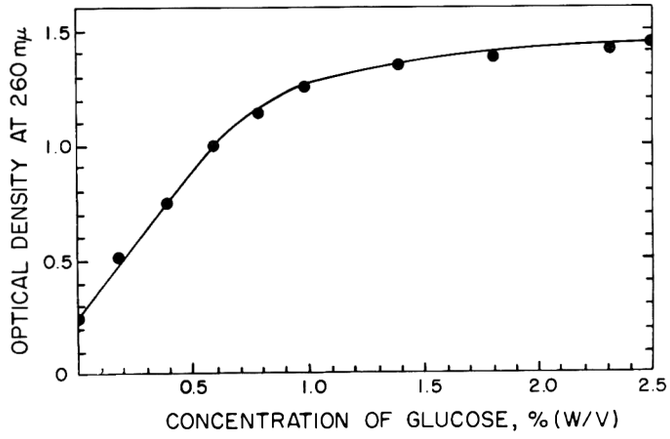

Fig. 2. Leakage of nucleotidic material from yeast L in response to the presence of glucose at various concentrations.

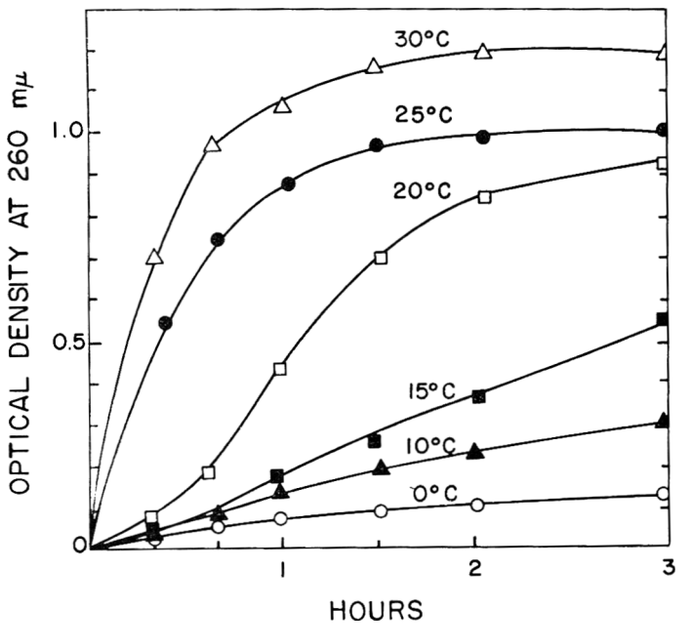

Fig. 3. Leakage of nucleotidic material from yeast L fermenting glucose solution at the temperatures shown.

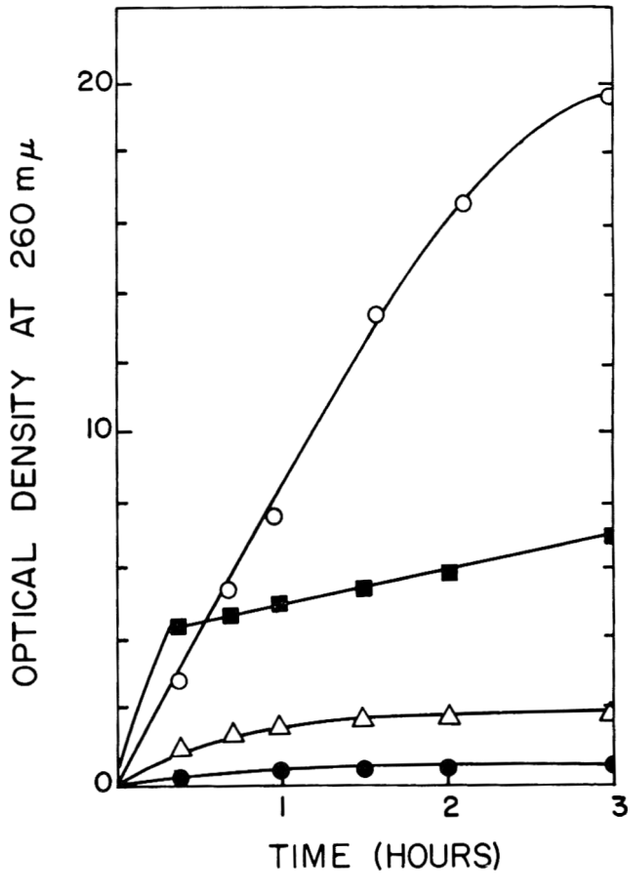

Fig. 4. Release of nucleotidic materials by yeast L suspended in water or sugar (10% w/v) solution with n*-butanol (3.2% w/v) and/or Ca^{++} (0.02M) added. Release in: ○—glucose + butanol. ■—glucose + butanol + Ca^{++}. △—glucose. ●—glucose + Ca^{++}; or lactose + butanol; or water + butanol.*

Mechanism of release

Some evidence was presented in the preceding section to show that the source of the released nucleotidic material was the intracellular free pool of these materials. Evidence presented in this section will support that contention by showing that the leakage is dependent on the condition of the cytoplasmic membrane.

Aldous (1965) has shown that the pH of the suspending medium can have some effect at the outer surface of the cell. In particular, adverse pH can damage the cytoplasmic membrane. Yeast was suspended in glucose solution containing phosphate buffer (0.1 *M*) at pH 2.0 through 8.0. Leakage of nucleotides was followed in the usual way (Table 3). At a "normal" pH for growth of this yeast (pH 4.0 or 5.0) the release was substantially the same as control. At pH 7.0 or 8.0 leakage was increased in the presence or absence of glucose solution. At pH 2.0 or 3.0, on the other hand, a substantial increase in leakage was found in the presence, but not in the absence, of glucose.

Rose (1963) and Dixon *et al.* (1964) reported that *n*-butanol damaged the cytoplasmic membrane of yeast. It seemed plain that if nucleotidic material leaked from a free pool, and if leakage was dependent on membrane integrity, then a weakened membrane should permit greater leakage. As shown in Fig. 4 this was found to be the case. Yeast suspended in glucose solution containing 3.2% w/v of *n*-butanol, leaked to a considerable extent, but leakage was rather negligible at this concentration of *n*-butanol in the absence of glucose. If lactose (unfermentable sugar) was substituted for glucose, *n*-butanol did not increase leakage appreciably. This showed that the osmotic pressure of the medium was not responsible for the data in Fig. 4. Similar results to those of Fig. 4 were obtained when a detergent (Triton X-100) or RNase (Schlenk *et al.*, 1965) was used as a membrane-damaging agent, except that leakage in the absence of glucose was somewhat increased.

Table 3. Effect of pH of the suspending medium on the release of nucleotidic substances by yeast fermenting glucose solution.[1]

Treatment		In glucose	In water
Normal pH	4.0	1.4	0.2
	5.0	1.4	0.2
Low pH	2.0	2.8	0.4
	3.0	3.0	0.3
High pH	7.0	2.4	2.3
	8.0	2.6	2.5
Control (no buffer)		1.4	0.2

[1] Leakage is reported after 1 hr of fermentation.

The association of calcium ions with the surface of yeast cells is well documented. Schlenk *et al.* (1965) for example showed that there was some association of Ca^{++} with the cytoplasmic membrane. This association has been used to protect the membrane from the action of polyene antibiotics (Stachiewicz *et al.*, 1963) or RNase (Schlenk *et al.*, 1965). It was found that Ca^{++} was effective in preventing the release of nucleotidic material by yeast suspended in glucose solution (Fig. 5). Calcium ions were equally effective in halting leakage whether added at the beginning of an experiment or after release of the nucleotidic material had already started. Mg^{++} had much the same effect as Ca^{++} but at higher concentrations. Ca^{++} was also partially effec-

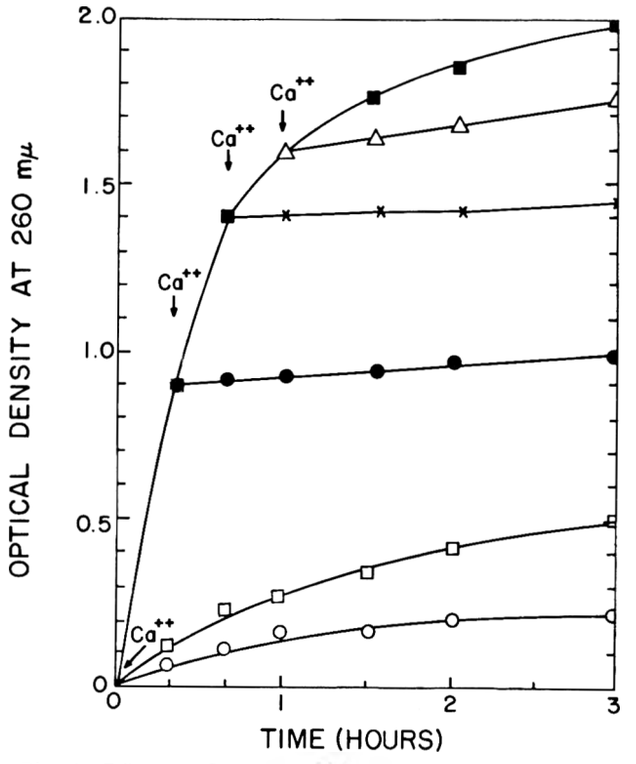

Fig. 5. Effect of Ca^{++} (0.02 M) on the release of nucleotidic material by yeast L suspended in glucose solution at 25°C. Symbols: ■—no Ca^{++} added. ○, ●, × △ — Ca^{++} added at 0, 30, 40, 60 min respectively. □—Leakage by yeast suspended in water.

tive in protecting the cell from the action of butanol in the presence of glucose (Fig. 4), indicating an effect at the cytoplasmic membrane.

Leakage of nucleotides is stimulated or induced by the presence of glucose. If the membrane is protected by Ca^{++} or Mg^{++} then glucose-induced leakage is halted. If the membrane is damaged by acid pH or *n*-butanol then glucose-induced leakage is increased. It was concluded that glucose-stimulated leakage was primarily a membrane phenomenon and is a result of an increase in membrane permeability (cf. Lewis *et al.*, 1967). This being so, the most obvious source of the leaking material was the free intracellular pool of nucleotides as shown in the preceding section.

DISCUSSION

Spiegelman *et al.* (1955) demonstrated that yeasts contain a free pool of nucleotidic material analogous to the free pool of amino acids. These pools were located close to the cell wall in the region of the ribosomes. When yeast is suspended in aqueous solution, these free pools are retained against a concentration gradient, in part possibly by the expenditure of energy but mainly through the presence of the cytoplasmic membrane (Mitchell, 1959).

Evidence is presented in this paper to show that the glucose-induced release of nucleotidic materials involves the free pool and the cytoplasmic membrane. This was a reasonable biochemical juxtaposition—the cytoplasmic membrane separates the internal pool of nucleotides from the medium. Little leakage occurred when the yeast was suspended in water, but in the presence of glucose, substantial leakage was observed. If the permeability of the cytoplasmic membrane was increased by the presence of membrane-damaging agents, then glucose-induced leakage was increased.

In the presence of a membrane-protecting agent, glucose-induced leakage was reduced to low levels. It seemed plain, therefore, that glucose-induced leakage was a phenomenon involving membrane permeability associated with the transport or utilization of glucose. This being so, it is assumed that the leakage is a normal physiological function of healthy cells under fermentative conditions.

Such leakage may be of consequence in the brewing and wine making industries since the released material was not reabsorbed during the course of an experiment and should persist to the finished product (Lee *et al.*, 1968). Under conditions of a commercial fermentation of brewers wort or a grape must, the high initial sugar concentration can lead to substantial accumulations of alcohols and esters. As butanol does, these organic solvents may affect the permeability of the cytoplasmic membrane in the presence of fermentable sugar leading to increased leakage. Phenethyl alcohol, for example, a component of beer and wine produced during fermentation, has recently been shown to damage the cytoplasmic membrane of *E. coli* leading to loss of cell contents (Silver *et al.*, 1967). During the later stages of a commercial fermentation, therefore, sugar-induced leakage of yeast cell contents may proceed apace. Lewis (1964a) estimated that about 50% of the U.V.-absorbing (260 mμ) materials of beer may originate in the yeast cell, which as shown in the preceding paper would account for most of the nucleotidic material of beer.

Yeast is well known to make a major contribution to the flavor of beer and wine. The involvement of this nucleotidic material in beer and wine flavor is under investigation.

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Identification of Some Sugars and Mannitol in Celery

SUMMARY—The carbohydrate content of celery petioles was determined using paper chromatographic techniques. Sucrose, glucose, fructose and mannitol were identified and quantitatively determined. Mannitol crystals were isolated. Sugars chromatographed with solvents containing boric acid showed characteristic stabilities to indicators.

INTRODUCTION

PROCESSING STUDIES in progress in this laboratory have created a need for more detailed knowledge of the carbohydrates in celery. Obaton (1929) reviewed the properties and occurrence of mannitol and found the polyol in all parts of the celery plant; it was thought to be synthesized in the leaves, transported through the petioles and stored in the roots. He lists Hubner *et al.* (1823) as the first report of mannitol in celery. Later, Payen (1934) described the isolation of the sugar from celery juice but gave no quantitative data.

Total and reducing sugars in celery petioles were determined by Hall (1957, 1959) among others. Siegel *et al.* (1962) determined glucose, fructose and sucrose in leaves from celery plants grown in a nutrient culture. In a recent review, Crosby *et al.* (1963) reported that only sucrose, glucose and apiose have actually been identified in celery, but he proposed that other common sugars were undoubtedly present. They mention one report (Anon., 1923) of mannitol having been found in celery roots and two reports of reducing sugars (Hall *et al.*, 1961, Myers *et al.*, 1921).

In view of the omission of the easily detectable mannitol in recent reports, it seemed prudent to reexamine the relative amounts of the predominant sugars in the commercially important celery petioles.

EXPERIMENTAL METHODS

MATURE FRESH PASCAL CELERY petioles from three plants were trimmed at both ends, washed, dried, and chopped into 3/8-inch chips. Four hundred g of the diced celery was blended with 2 g calcium carbonate and the resulting slurry was heated 1 hr on a steam bath. After filtering through a coarse paper filter with suction, the residue was mixed with 100 ml water, heated 1 hr on a steam bath and filtered. The extraction was repeated four more times and the extracts were combined.

A final filtration of the combined extracts was done with a medium porosity sintered glass filter which had been coated with an aqueous suspension of Analytical Grade Celite and washed with water. The solution was made to a known volume and deionized with Dowex 50W-X4 and Duolite A4 ion exchange resin similar to the procedure of Partridge (1948). Aliquots were chromatographically analyzed for all sugars. Proteins, if extracted, did not interfere.

Whatman No. 1 chromatographic paper was used as received for the identification of the sugars. All chemicals were reagent grade unless otherwise specified, and all melting points are corrected.

The sucrose, glucose and fructose bands were identified by their colors and reaction times when sprayed with *p*-anisidine hydrochloride (Hough *et al.*, 1950) and aniline hydrogen phthalate (Partridge, 1949). Sucrose was confirmed by its reaction with invertase (Williams *et al.*, 1951) and glucose, by elution, oxidation to gluconic acid and isolation of the potassium salt (m.p. 180°C) (Moore *et al.*, 1940).

Mannitol, sorbitol, and in some cases fructose will react feebly or not at all with silver nitrate after being developed in solvents containing water saturated with boric acid. Dedonder (1952) showed that n-butanol:ethanol:water (40:11:19) will separate glucose, fructose and sucrose. However, in this solvent, mannose and fructose had nearly the same R_f. They were distinguished by developing in n-butanol:ethanol:aqueous saturated boric acid (40:11:

19) and using silver nitrate as the indicator. Fructose was confirmed by its stability to silver nitrate when developed in this solvent. Similarly, the polyol spots in n-butanol: acetic acid:water (4:1:5) were stabilized to the silver nitrate indicator by using aqueous boric acid.

Schleicher and Schuell 2043b paper was used for the quantitative determinations of the sugars. The trailing edge of the paper was serrated and the paper was washed overnight in the solvent before being used. The chromatograms were developed 18–20 hr by descent in a closed tank at room temperature. The spots were detected by dipping the paper in an acetone solution of silver nitrate, drying, dipping in ethanolic sodium hydroxide, drying and dipping in saturated 60% ethanolic thiosulfate (McCready *et al.*, 1966). The concentrations were determined with a Photovolt Densitometer and deionized standards, using the method of McFarren *et al.* (1951).

Of the sugars tested, only mannitol and sorbitol did not react with silver nitrate when a chromatogram was developed in the solvent used by Rees (1958) (2-butanone: acetic acid:aqueous saturated boric acid, 9:1:1). This stability persisted when ammoniacal silver nitrate (Partridge, 1948) or alkaline permanganate (Pacsu *et al.*, 1949) were sprayed, but the sugar alcohols did react if the dried chromatogram was heated to 100–110°C for 10–15 min and immediately immersed in the acetone-silver nitrate solution.

To isolate mannitol, dried celery (*ca.* 3% moisture) was blended with petroleum ether (Skelly B) and filtered. The residue was refluxed 2 hr with 95% ethanol and filtered hot. On cooling, grayish crystals of mannitol precipitated. The mannitol was recrystallized three times by dissolving it in a minimum amount of water, filtering, and adding two volumes of 95% ethanol.

The identification of the crystalline isolate as mannitol was based on the following observations. The dried colorless crystals melted at 166°C. The hexa-acetate prepared by the method of Shriner *et al.* (1956) melted at 119–120°C and had (a) D^{25} +25.2° in $CHCl_3$. The melting points and specific rotation agree with those stated by Heilbron *et al.* (1953). Crystallographic comparison by Dr. F. T. Jones of this laboratory of the unknown and its acetate with authentic samples confirmed the identification.

RESULTS

THE WATER EXTRACT of celery was chromatographed against known standards and the following average concentrations were determined (g/100 g fresh celery): sucrose 0.488, glucose 0.309, fructose 0.314 and mannitol 0.114. The results are the average of three determinations. Maximum variation between determinations of a sample was 4.1%. The sensitivity of the experimental procedures precluded detection of sugars such as apiose which are present in small amounts.

As mentioned earlier, the sugars were extracted from the petioles with water. Although this solvent insured the solubilization of mannitol, it may have permitted invertase activity. Therefore, the sucrose value might be low even though it is higher than some of the literature reports. The high solubility of mannitol in hot alcohol would have complicated the use of boiling alcohol for enzyme inactivation before the quantitative determination.

Obaton (1929) determined mannitol, reducing sugars and sucrose at 10 maturity levels in the variety "Celeri rave de Paris amélioré." He found the following concentration ranges (g/100 g fresh weight): mannitol 0.75–2.93, reducing sugars 0.21–1.10, and sucrose 0.1–0.4.

The results of Myers *et al.* (1921) for reducing and non-reducing sugars are within the above ranges.

Since different varieties would not necessarily contain similar sugar concentrations, the results mentioned previously might not be comparable to the more recent work.

The results of Hall *et al.* (1961) are typical of some of the more recent sugar determinations. He examined Florida Summer Pascal celery petioles at different maturity levels and found the following concentration ranges (g/100 g fresh weight): inner petioles, reducing sugars 0.8–1.3 and total sugars 0.9–1.6; outer petioles, reducing sugars 0.8–1.2 and total sugars 0.9–1.5. The chromatographically determined sugar concentrations are within this range. The tests used for reducing sugars would not be expected to detect the polyols.

Hall (1959) suggested that the sugar content appears to be an important part of the total flavor of celery petioles. Since Carr *et al.* (1936) and Schutz *et al.* (1957) found d-mannitol to be about 60–70% as sweet as sucrose, or about as sweet as glucose, and the polyol is present in large amounts, it appears that mannitol is an important constituent of celery petioles.

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Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

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Serological Identification of Animal Proteins. 1. Mode of Injection and Protein Extracts for Antibody Production

SUMMARY—Specific antibodies were developed against skeletal muscle from horse, pork, lamb and beef. The antigenic protein material evaluated for antibody production included actomyosin, serum-alum precipitate, muscle extract-alum precipitate, saline extract of muscle and freeze-dried water extract of muscle. The method of injection into the rabbits included intraperitoneal, intravenous, subcutaneous, and intramuscular with and without Freund complete adjuvant.

Of the antigenic protein material and route of injection evaluated, the intramuscular injection of 150 mg of freeze-dried water extract of muscle with Freund complete adjuvant resulted in the highest titers which were observed as the titer increased and with time after injection as indicated by ring and gel diffusion tests. However, these cross-reactions could be removed by absorption with small amounts of the freeze-dried protein extracts of the cross-reacting species. A specific antiserum for each animal specie could be obtained which would react with 0.4–0.5 mg/ml of protein in a saline extract of skeletal muscle.

Overall, multiple intramuscular injections of freeze-dried water extracts of skeletal muscle emulsified in Freund complete adjuvant resulted in the highest titers which would react specifically with each animal species.

INTRODUCTION

IDENTIFICATION OF SKELETAL MUSCLE as to species of animal when present in a ground meat mixture is time consuming and usually inconclusive when identified by chemical techniques. The adulteration of ground meat or processed meat items by adding horsemeat or other meat which has not been properly declared on the ingredients list is not common but has been known to occur. A simple, rapid test would make it possible to determine the addition of undeclared meat in a product and give the consumers greater protection.

The injection of a foreign protein (antigen) into an animal's tissue fluid will produce blood soluble substances known as antibodies. These circulating antibodies in the globulin fraction of blood serum will react specifically with the specific foreign protein either *in vivo* or *in vitro*. Oswald (1953) demonstrated that antibodies could be formed against the desired species by injection of an alum-serum precipitate of that species. However, results obtained in this laboratory using this procedure were not entirely satisfactory as to level of detection or specificity.

The purpose of this study was to investigate various antigen preparation and routes of injection to determine the optimum method for producing antibodies of the desired titer and specificity.

EXPERIMENTAL PROCEDURES

Preparation of antigenic material

Actomyosin. Ground, frozen, lean, skeletal muscle from horse, beef, lamb or pork was thawed at 4°C overnight. The lean tissue was blended at low speed in an Osterizer with .6 *M* KCl at a ratio of 1 part tissue to 3 parts KCl for 3 min. The resulting slurry was centrifuged at 5°C for 15 min at 10,000 × G. The supernatant was removed and diluted 10 fold with distilled water and allowed to set for 15 hr at 4°C. The actomyosin which precipitated was removed by centrifugation at 1,400 × G at 5°C for 15 min. The identical precipitation and centrifugation procedures were repeated three more times. After the last washing, the precipitate of actomyosin was suspended in a small quantity of distilled water and lyophilized. The dried actomyosin was stored in an airtight container at −20°C until used.

Serum. Blood was collected from the particular species and allowed to clot. The clotted blood was held overnight at 4°C and the serum removed by centrifugation at 1,400 × G for 30 min at 5°C. The serum was stored at −20°C in sealed test tubes until used.

Serum alum precipitate. Serum was collected as described previously. Twenty-five ml of the serum was diluted with 80 ml of distilled water and 90 ml of 10% potassium alum was added. The pH was adjusted to the isoelectric point of the particular species of serum used (beef 5.6) with 5 *N* NaOH (approx. 3 ml) and allowed to stand for 3 min. The precipitate was separated by centrifugation at 5,000 × G for 15 min at 5°C. The precipitate was then washed twice with 200 ml portion of 1:10,000 merthiolate physiological saline solution by repeated centrifugation. The precipitate was made up to a total volume of 100 ml with 1:10,000 merthiolate physiological saline and frozen at −20°C in small aliquots until used. This procedure is similar to the one described by Oswald (1953).

Muscle extract alum precipitate. Ground, frozen, lean, skeletal muscle was thawed at 4°C overnight. The lean tissue was extracted with distilled water (1 part tissue to 2 parts water) by blending at low speed in an Osterizer for 15 min. The slurry was centrifuged at 10,000 × G for 15 min at 5°C and the supernatant filtered through No. 4 Whatman filter paper. Ninety ml of 10% potassium alum was added to 25 ml of the supernatant. The pH was adjusted to the isoelectric point with 5 *N* NaOH and allowed to set for 30 min. The precipitate was separated and washed as previously described above for serum alum. The final precipitate was brought to a total volume of 100 ml with 1:10,000 merthiolate saline. The suspension was frozen in small aliquots at −20°C until used.

Saline extract. Ground, frozen, lean, skeletal muscle was thawed overnight at 4°C. The tissue was blended at low speed in an Osterizer with physiological saline (1 to 4) for 15 min. The slurry was centrifuged at 10,000 × G for 15 min at 5°C and the supernatant filtered through

No. 4 Whatman filter paper. One ml of 1% merthiolate was added per 100 ml of extract. These extracts were stored no more than 3 days prior to injection.

Freeze-dried water extracts. Ground, frozen, skeletal muscle was thawed at 4°C overnight. The tissue was extracted with distilled water (1 to 3) by blending at low speed in an Osterizer for 30 min. The slurry was centrifuged at 10,000 × G for 15 min at 5°C and the supernatant filtered through No. 4 Whatman paper. The extract was lyophilized and the dried extract placed in an airtight container and stored at −20°C until used.

Serological methods

Antiserum preparation. Blood was removed from the rabbits by bleeding from either the marginal vein of the ear or by cardiac puncture. The blood was allowed to clot at room temperature and placed in a 4°C cooler for 18 hr. The antiserum was decanted and centrifuged at 1,000 × G for 30 min to remove any remaining blood cells present. The antiserum was transferred to test tubes and merthiolate added at a final concentration of 1:10,000 and stored at 4°C.

Absorbed antiserum was prepared by adding a small amount (approx. 8 mg/ml) of freeze-dried antigenic protein of the species which were cross reacting. The antiserum was shaken by an Eberback Shaker at room temperature for 4 hr and placed in a 4°C cooler for 14 hr. The antiserum was centrifuged at 2,000 × G to remove the precipitate containing the cross reacting antibody-antigen complex.

Preparation of test antigens. A 50 g sample of skeletal muscle from either horse, beef, pork or lamb was blended with 150 ml of physiological saline at a low speed in an Osterizer for 30 min. The slurry was centrifuged at 10,000 × G for 15 min. The supernatant was filtered through No. 4 Whatman filter paper and was stored for no longer than 4 days.

Ring test. A 4 × 50 mm precipitation tube was filled approximately half full with the antiserum using a syringe and needle. The antigen was carefully layered over the antiserum using a syringe and needle in such a way that two distinct layers were formed. Tubes were examined in 15 to 30 min using an artificial light against a black background. If the antigen and antiserum reacted, a precipitate was formed as a white ring at the interface of the two solutions as shown in Fig. 1. The density of the precipitation ring was recorded in the following way: + slight, ++ moderate, +++ strong, ++++ very strong and 0 no reaction.

Gel precipitation. The double diffusion method (Ouchterlony, 1949) was used. Oxoid Ionagar No. 2 (0.85%) was dissolved in borate buffered saline at a pH of 8.4 and merthiolate added to a final concentration of 1:10,000. The agar was autoclaved for 10 min at 15 psi and poured to a depth of 9 mm in 15 × 50 mm petri dishes. Seven wells were cut 2 cm apart in the agar as indicated in Fig. 2. The bottom of the well was sealed with a drop of hot agar. From 0.1 to 0.3 ml of the reactants were placed in the wells and the plates were stored in a moist desiccator for 3 days at room temperature. The precipitation bands were observed and recorded.

Wheal test. The hair was removed from a small area on the side of the rabbit. A small amount (0.1 to 0.2 ml) of the test antigen was injected subcutaneously into a rabbit which had been previously injected for antibody production. A positive test was observed when the test area swelled and developed edema of varying intensities within ½ to 6 hr after which the reaction faded.

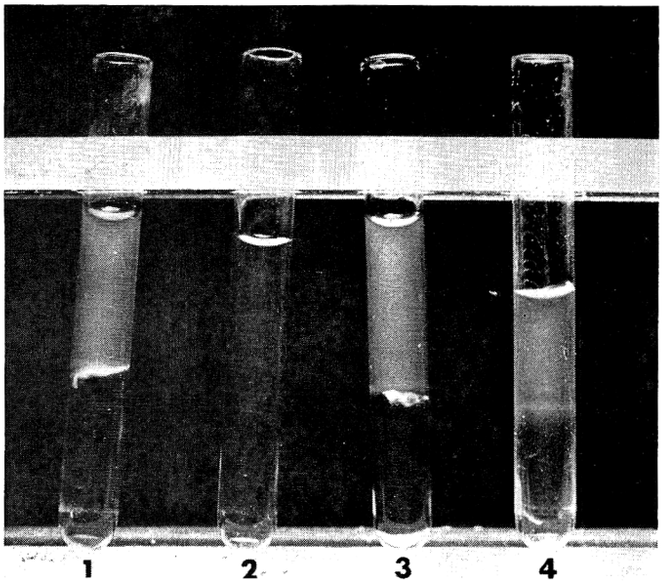


Fig. 1. Example of ring test of beef antiserum with beef extract: 1. Rabbit No. 43 = ++++; 2. Rabbit No. 51 = 0; 3. Rabbit No. 34 = +++; 4. Rabbit No. 48 = +.

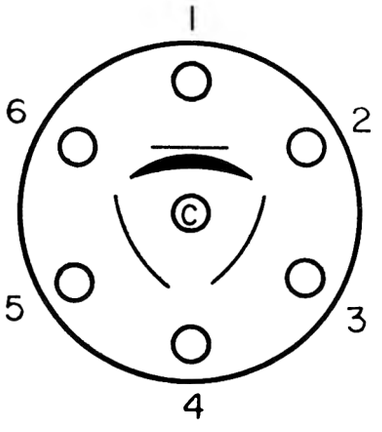


Fig. 2. Precipitation bands of unabsorbed beef antiserum on gel diffusion plates.

RESULTS AND DISCUSSION

SEVERAL IMPORTANT FACTORS are involved in developing specific antibodies against skeletal muscle which are different from bacterial or serum antigens. The quantity of antigenic protein material, the route of injection and type of antigenic protein material are all important.

The rabbits were injected intraperitoneally with 2 ml of a saline tissue extract on alternate days for eight injections and were rested for one week and seven additional injections were given on alternate days. The protein content of the extract was 10 to 15 mg/ml as determined by the Kjeldahl procedure (AOAC, 1965). The rabbits were bled after 10 days from the marginal vein of the ear. A slight positive reaction was obtained with the ring test for rabbits injected against the homologous antigen. However, when gel diffusion tests were run on the same antiserum the results were negative against the homologous and heterologous antigens.

Similar results were obtained when the rabbits were bled after 30 days. After 60 days negative ring and diffusion tests were observed. However, when the rabbits were injected intravenously with 5 ml of saline tissue extract after 120 days, the rabbits went into anaphalaxis shock and died within 4 hr. This would indicate that antibodies were present but the titer was not high enough to be detected by ring or gel diffusion tests.

Actomyosin was isolated from muscle tissue using the procedure of Szent-Gyorgyi (1951) and evaluated as one of the antigenic materials. Initially, 10 mg of actomyosin of a particular species was suspended in 1 ml of Freund complete adjuvant and 0.5 ml injected intramuscularly into each hind leg of the rabbit. After one month ring tests against saline tissue extracts of each species were negative as were 0.6 *M* KCl extracts.

The same rabbits were given a second injection of 20 mg of actomyosin rehydrated in 1 ml of 0.6 *M* KCl and emulsified in 1 ml of Freund complete adjuvant and 1 ml injected intramuscularly into each hind leg 45 days after the first injection. Negative results were obtained for ring tests and gel diffusion tests after 20 days from the last injection against tissue extracts of each species. In addition, another group of rabbits was injected subcutaneously at 10 locations along the spinal cord with 0.2 ml of the same preparation of antigenic material. These also gave negative results for ring and gel diffusion tests after 30 and 60 days.

The same group of rabbits was given one final injection of 150 mg of actomyosin rehydrated in 2 ml of 0.6 *M* KCl and emulsified in 2 ml of Freund complete adjuvant. Two ml were injected intramuscularly into each hind leg. A slightly positive ring test was observed when bled after 20 days in five out of the six rabbits tested. However, all rabbits gave negative ring tests after 60 days. Wheal tests using the same antigenic material as previously injected showed a positive reaction varying from a moderate to strong amount of edema in the area injected after 4 to 6 hr. From these results, it was concluded that the amount of antigenic material injected must be considerably higher than that required for bacterial or serum antigens, at least, in the case of actomyosin.

Another group of five rabbits was injected intraperitoneally with beef actomyosin (150 mg) suspended in 15–20 ml of physiological saline. Injections were on alternate days of four injections, rested for 10 days and two more made one day apart. After 20 days, the rabbits were bled by cardiac puncture and then at weekly intervals for 9 weeks. A slightly positive ring test was observed for beef extract and beef serum for six weeks after the initial bleeding. In many cases, a slightly positive reaction was obtained for horse, pork and lamb extracts, indicating cross reactions were occurring. However, when the same antiserums were tested in gel diffusion plates a positive precipitation band was observed only with beef serum in four out of five rabbits. After six weeks, no reaction was observed for any antigen tested by either the ring or gel diffusion test. Actomyosin did not produce antiserum of sufficient specificity or titer to be used in detecting species differences.

Injection of diluted beef serum (1:4) for 9 consecutive days resulted in positive tests for beef serum but negative for all tissue extracts by ring or gel diffusion tests. Similar results were obtained with a single injection of 3 ml of beef serum per leg, with the exception, that a strong positive ring test was observed after 5 days with beef tissue extracts. However, 13 days later negative ring and gel diffusion tests were observed for all tissue extracts.

Since a strong initial response was observed with injection of beef serum, another group of rabbits was given a series of injections with a rest period to take advantage of the initial and secondary antibody response. The rabbits were given intramuscular injections of 5 ml of undiluted beef serum per leg for four injections on alternate days, rested for 10 days and given two more injections. Fourteen days later the antiserum showed a strong, positive reaction with the ring and gel diffusion tests for beef serum. Similar strong reactions were observed over a 90 day period when antiserum was collected on a weekly basis. Ring tests with tissue extracts were only slightly to moderately positive for beef and a slightly positive reaction for horse and lamb tissue extracts during some of the weekly test bleedings. Gel diffusion plates had a precipitation band for beef, horse and lamb extracts. However, when cross reacting antibodies were absorbed out by the addition of cross reacting antigens, no precipitation bands were observed with beef extracts though the beef serum still gave a precipitation band. It was concluded that the injection of serum would result in the formation of antibodies which lasted over an extended period of time. However, the reaction with tissue extracts was not strong enough or specific enough for detecting species difference of skeletal muscle.

The procedure of Oswald (1953) was followed in the preparation and injection of beef serum alum. The antigenic material was prepared and 5 ml was injected intramuscularly per leg. Fifteen days after the injection, a strong positive ring test for beef serum was observed. However, only a slightly positive test was observed with beef muscle extracts and horse muscle extracts. Similar results were observed with beef muscle extracts and horse muscle extracts. Similar results were observed on the gel diffusion plates as indicated by the precipitation bands. However, due to cross reactions and inability to obtain

high titers over a period of time, this procedure was terminated.

Tissue extracts were prepared and precipitated using alum in a method similar to that described by Oswald (1953) for serum. One or two injections of beef extract alum per leg gave very little detectable antibody response as determined by ring and gel diffusion tests. Therefore, multiple injections in two series were used in order to develop higher titers. Rabbits were injected intramuscularly with 5 ml of alum precipitated tissue extract per leg on alternated days for four injections, rested 10 days and given two subsequent injections in a similar manner. Fifteen days after the last injection, ring tests for beef extract were only slight to moderately positive; however, the gel diffusion tests were positive for beef extracts, beef serum and cross reacted with lamb and horse extracts. Antibodies were detected for at least 90 days using ring and gel diffusion tests. However, when the cross reacting antibodies were absorbed using freeze-dried tissue extracts of the cross reacting species, it was no longer possible to detect antibodies for the specific species.

Since the previous work had shown the necessity of injecting relatively large amounts of protein material, skeletal muscle was extracted with distilled water and freeze-dried as described in the procedure. Initially 150 mg of the freeze-dried extract was dissolved in 2 ml of physiological saline and injected intraperitoneally on alternate days for 4 days into the rabbits, rested for 10 days and then two additional injections were given one day apart. After 14 days a moderately positive ring test for beef serum and slightly positive test for beef extract was obtained. After 28 days, a strong reaction was observed for beef serum and beef extract and a slight reaction for horse and lamb extracts. Similar results were observed at intervals over a 90-day period.

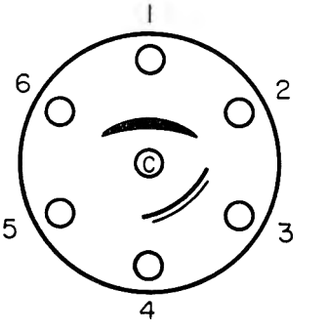
Gel diffusion plates showed only a slight precipitation band after 14 days for beef serum. After 28 days, a strong precipitation band was observed with beef serum and beef extract and a slight precipitation band with horse and lamb extracts. However, these cross reactions could be removed by absorption with a small amount of freeze-dried antigens. Specific reactions were observed only with beef serum and beef tissue extracts on gel diffusion plates. These reactions were also observed when the antiserum was diluted 1:5 with saline, but the precipitation bands were not as heavy. In addition, the amount of cross reaction increased after 60 days but could be removed by absorption with freeze-dried antigens. With this method a specific antigen-antibody reaction could be produced and could be repeated from week to week.

At the same time, a second group of rabbits were injected intramuscularly using 150 mg of freeze-dried beef extract dissolved in 2 ml of physiological saline and emulsified in 5 ml of Freund complete adjuvant. Three and one-half ml were injected into each hind leg using the same intervals as the interperitoneal injections for rabbits. The results were similar to those obtained with the intraperitoneal injections except that the reactions were stronger for both the ring and gel diffusion tests. This method gave the strongest reactions and continued for up to 120 days.

This method was then selected for additional tests using 20 rabbits with freeze-dried tissue extracts from horse, lamb, beef and pork. Two intramuscular injections of 150 mg of the freeze-dried tissue extract dissolved in 2 ml of physiological saline and emulsified in 5 ml of Freund complete adjuvant were given one day apart. The rabbits were rested for 22 days and two more injections were given in the same way as the initial injection. After 21 additional days, the ring test ranged from a slight to strong reaction for the specie injected. Cross reactions were observed particularly between beef and lamb. The rabbits were bled on weekly intervals and the number of strong positive reactions increased up to at least 120 days. Specific antiserums with high titers were obtained by absorption with freeze-dried antigens of cross reacting species.

The more sensitive gel diffusion test was used to evaluate antigen-antibody reactions, cross-reactions and titer of specific antiserums obtained. Beef antiserum reacted with beef serum and beef extract as shown in Fig. 2. Usually two definite precipitation bands were observed between beef antiserum and beef extract and beef serum and one precipitation band between beef antiserum and lamb extract. The beef antiserum was absorbed with small amounts of lamb, horse and pork freeze-dried extracts. This absorbed beef antiserum showed only precipitation bands for beef extract and beef serum as shown in Fig. 3. This reaction was observed even when the absorbed antiserum was diluted 1:8 with physiological saline though the precipitation bands were more diffused. When the beef muscle extract was serially diluted as shown in Fig. 4, a precipitation band was shown at a dilution of 1:128 which corresponded to a protein content of 0.5 mg/ml.

Horse antiserum was specific initially but tended to cross react with other species after 90 days. However, these cross reactions could be removed by absorption with freeze-dried extracts of other species. All other results were similar to those observed with beef. It was possible to detect 0.4 mg/ml of protein in diluted extracts.



C ABSORBED BEEF ANTISERUM
1 BEEF SERUM
2 PORK EXTRACT
3 BEEF EXTRACT
4 HORSE EXTRACT
5 LAMB EXTRACT
6 SALINE

Fig. 3. Precipitation bands of absorbed beef antiserum on gel diffusion plates.

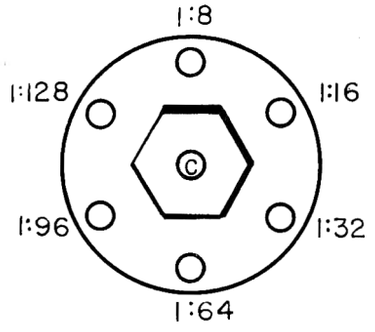


Fig. 4. Dilution plate of absorbed beef antiserum.

The results observed with pork were similar to those obtained with horse. Initially, the pork antiserum was very specific but after 90 days cross reacted with other species. These cross reactions could be removed by absorption with freeze-dried extracts. A positive precipitation band was obtained when extracts were diluted to a protein content of 0.4 mg/ml.

The lamb antiserum cross reacted initially with beef extracts and after 90 days with horse extract. These cross reactions could be removed by absorption to obtain a specific lamb antiserum which would react with lamb extracts diluted to 0.4 mg/ml. All other reactions were similar to those observed with beef.

CONCLUSIONS

Intramuscular injections of freeze-dried skeletal muscle extracts with adjuvant will cause antibodies to be produced which can be made to react specifically with saline extracts of that muscle. This injection procedure and antigenic protein material was superior to saline extracts, actomyosin, serum, serum-alum precipitate and extract-alum precipitate.

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Ionizing Radiation Effects on Starch as Shown by Staudinger Index and Differential Thermal Analysis

SUMMARY—Corn starch in the form of raw granules at commercial moisture was irradiated at two levels: 3×10^6 and 6×10^6 rad from a Co^{60} source. The irradiated samples were completely dissolved in alkali, indicating there was no cross linking induced in the starch molecules by irradiation. Viscosity determinations of starch solution diluted with distilled water exhibited the ion charge effect generally observed in other macromolecules.

The Staudinger indices of unirradiated, irradiated at 3×10^6 rad and 6×10^6 rad were 42, 22 and 16 respectively, which were an indication of depolymerization of starch macromolecules with increasing irradiation. The differential thermal analysis of the three samples also showed the depolymerization of the polymer with irradiation.

It is suggested that these two simple techniques—the Staudinger index and D.T.A.—could be usefully employed to characterize small differences in starches.

INTRODUCTION

DUE TO THE IMPORTANCE of starch in the food and textile industry, considerable work has been done to investigate the structure, properties and use of this polysaccharide. Research in the last two decades on the ability of ionizing radiation to kill micro-organisms without the use of heat suggests a means by which heat sensitive cereal products may be pasteurized to increase their shelf life. However, as a direct result of ionizing radiation, a number of changes affecting the properties of the starches may occur.

To determine the gel-forming power and plastic strength of starches various kinds of viscometers have been used. However, because of the difficulty of getting a clear solution of starch, only limited investigations have been made using the Ostwald viscometer. Also differential thermal analysis has become increasingly important in the polymer field. In the work reported here, these two techniques were used to obtain evidence of molecular changes brought about in starch by ionizing radiation.

LITERATURE REVIEW

Brownell *et al.* (1955) working with irradiated bread and cake flours concluded that treatments up to 2×10^4 rep did not change the baking properties of the flours. However, cakes made from flours receiving higher dosages had low total volumes, were heavy, compact and had a yellow crumb color. Bauman *et al.* (1957) showed that with irradiation of cake batters using energy up to 2×10^6 rep, gelatinization properties of the starch were markedly changed. The gelatinization characteristics of the starch were also changed in dry cake mixes at a high level of irradiation.

Miller *et al.* (1965), stated that white cake and bread made from flour irradiated with 2×10^4 to 5×10^4 rad are distinguishable and less palatable than similar products baked from non-irradiated flour. Kertesz *et al.* (1959) have shown that with ionizing radiation starch macromolecule is depolymerized with the formation of low molecular weight dextrins and sugars.

Work by Wolfrom *et al.* (1959) demonstrated that carbohydrates underwent color and organoleptic changes on irradiation in the solid state. Irradiation of the disaccharides and trisaccharides showed that the glycosidic bond is especially sensitive to the action of ionizing radiation and that the order of hydrolysis is qualitatively that found with acid hydrolysis. Hydrolysis of the disaccharides and trisaccharides increased with increasing irradiation dosage.

MATERIALS AND METHODS

CORN STARCH in the form of raw granules at commercial moisture was irradiated at two levels i.e. 3×10^6 and 6×10^6 rad from a Co^{60} source. For the determination of viscosities, clear cold solution of starch was prepared. A 5.0 g sample of starch was moistened with 20 ml 95% ethanol. After 20 min 200 ml N NaOH was added and mixture allowed to stand for 30 min with occasional stirring. The mixture was then made slightly acidic with $2N$ HCl and back-titrated to neutrality with $0.01N$ NaOH. This solution was diluted to 500 ml to give a 1% starch solution. Further dilutions of 1% solution were made and viscosity determined with an Ostwald viscometer No. 100 at 24°C according to Jirgensons *et al.* (1962).

The Staudinger index $[\eta]$ is defined by the International Union of Pure and Applied Chemistry 1957 (Jirgensons 1958) as

$$[\eta] = \left(\frac{\eta - \eta_0}{\eta_0 \, C} \right)_{c=0}$$

where η and η_0 are the time required by the sample solution and the solvent respectively to flow equal distances in the viscometer, and C is the concentration expressed in grams of solute per milliliter. With concentration expressed in grams of solute per 100 ml, $[\eta]$ has long been known as the intrinsic viscosity.

For differential thermal analysis (DTA), samples were dried to a constant weight over P_2O_5 under vacuum. The samples were mixed with calcined alumina in the ratio of 1:3 and the DTA curves were obtained at the rate of 3°C per minute up to 500°C, with a rotating drum-type apparatus in the presence of air as described by Smothers *et al.* (1958).

A part of a thesis presented to the Graduate School in partial fulfillment for the requirements of the degree of Master of Science.

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Table 1. Total solids, sodium chloride and amount of starch in 500 ml of 1% solution (t = 24°C) (Average of triplicate readings).

Sample	Total solids g	Sodium chloride g	Starch g
Unirradiated	16.89	11.93	4.96
Irradiated 3×10^6 rad	17.16	12.16	5.00
Irradiated 6×10^6 rad	17.05	12.00	5.05

RESULTS AND DISCUSSION

IONIZING RADIATION is said to cross link some macromolecules but this was not observed in corn starch samples studied. The 500 ml 1% solution was centrifuged at 2500 rpm for 1 hr and top about 400 ml were retained for the investigations. Concentration of starch in solution was determined by drying 5 ml aliquot of the solution and subtracting the amount of NaCl determined by $AgNO_3$ titration in another aliquot. The results indicated that starch was completely dissolved and the variation was within the experimental error (Table 1). If these starch granules had been appreciably cross linked, the alkali used would not have dissolved the granules completely.

Viscosities were first determined by using distilled water for dilution of 1% solution. The viscosity number $\frac{\eta}{\eta_0}$ (formerly known as relative viscosity) decreased at first with increasing concentration of the starch solution and then decreased (Fig. 1). This ion charge effect observed was similar to that reported by Jirgensons *et al.* (1962) for polyacrylic acid. The effect was marked at lower levels of irradiation at 3×10^6 rad, but not so at 6×10^6 rad, which indicated that as the level of radiation increased the starch molecule is depolymerized such that it is less ionized.

To overcome the ion charge effect and to obtain straight line curves which could be extrapolated to C = O for the determination of Staudinger index, dilutions for the viscosity measurements were made with 0.4*N* NaCl, the concentration found in the 1% starch solution.

The Staudinger indices for the unirradiated, irradiated at 3×10^6 rad and 6×10^6 rad were 42, 22 and 16 respectively (Fig. 2). Since the Staudinger index $[\eta]$ is related to the molecular weight M of a polymer as

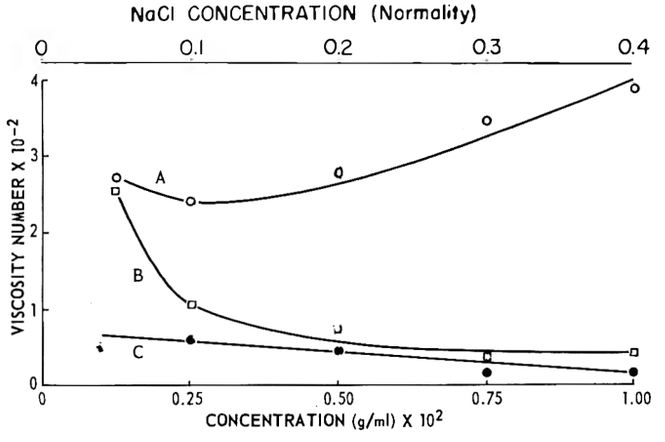

Fig. 1. Ion charge effect on viscosity number of starch solutions. A. Unirradiated. B. Irradiated 3 × 10⁶ rad. C. Irradiated 6 × 10⁶ rad.

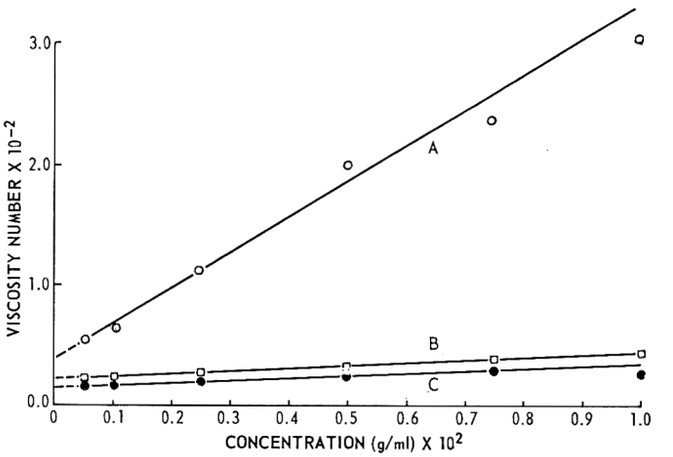

Fig. 2. Staudinger index of starch solutions. A. Unirradiated. B. Irradiated 3 × 10⁶ rad. C. Irradiated 6 × 10⁶ rad.

$$[\eta] = KM^a$$

where K and a are constants, these values indicated that with the increasing levels of irradiation, there was a decrease in molecular chain length.

The differential thermal analysis (Fig. 3) showed a slight exothermic reaction around 288°C followed by an endothermic reaction around 325°C and a second endothermic reaction at about 340°C in unirradiated starch. By contrast, irradiated starch showed only one endothermic reaction. It occurred around 275°C when irradiation was 3×10^6 rad and around 284°C with 6×10^6 rad irradiation. Thus irradiation caused chemical changes in the starch.

The cause of the endothermic reaction in the DTA

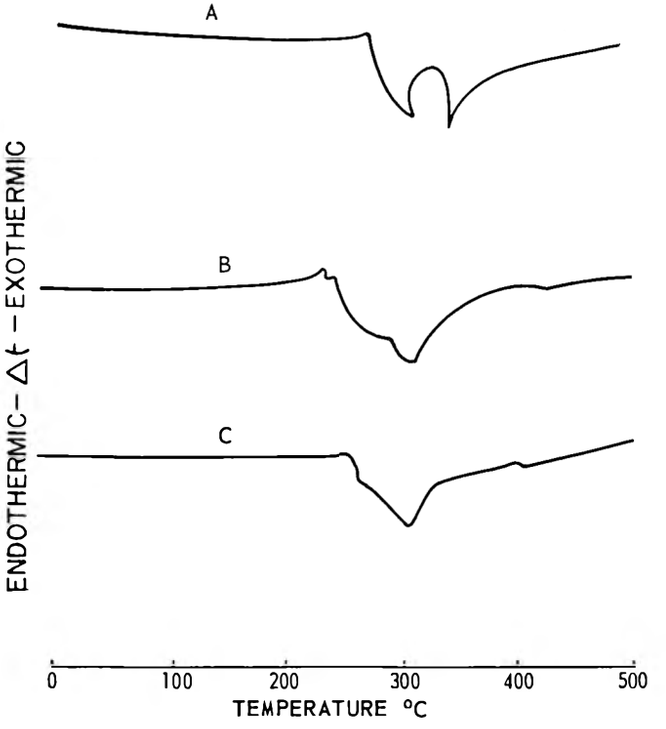

Fig. 3. Differential thermal curves of starch. A. Unirradiated. B. Irradiated 3 × 10⁶ rad. C. Irradiated 6 × 10⁶ rad.

curves presented in this study, shown by the deviation of the temperature line below the curve, is not known with certainty. It is probable that these reactions occur due to the loss of water in burning. The exothermic reactions seem to be due to oxidation.

CONCLUSIONS

Two simple techniques—the Staudinger index and DTA—could be usefully employed to characterize small differences in starches.

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Thermal Degradation of Black Raspberry Anthocyanin Pigments in Model Systems

SUMMARY—The thermal degradation of anthocyanin pigments of black raspberries as influenced by pH, oxygen, sugars and their degradation products were studied. The degradation of the major anthocyanin component (cyanidin-3-diglucoside), the total isolated pigments and the pigment in the natural berry juice was retarded as the pH decreased. Under the same conditions, cyanidin was much more unstable than any of the anthocyanin containing systems. In all cases studied, replacement of the oxygen atmosphere with nitrogen enhanced pigment stability. The rate constants for the thermal degradation of cyanidin-3-diglucoside at various pH levels under oxygen and nitrogen were determined. The sugars studied accelerated pigment destruction to the same extent. Sugar degradation products were more effective than sugars in accelerating anthocyanin breakdown.

INTRODUCTION

Loss of anthocyanin pigment is an important factor contributing to the color deterioration of various fruit products. Chemically anthocyanins are quite unstable, both in solution and in cellular media. They may easily change from their characteristic natural red or blue color to the undesirable brown-colored compounds. Many factors prevailing during processing have been shown to influence the degradation of the pigment. Considerable work on the principal anthocyanin of strawberries (pelargonidin-3-glucoside) revealed that pH, temperature and presence of oxygen are of paramount importance (Lukton *et al.*, 1956; Meschter, 1953).

Cellular constituents, including sugars and ascorbic acid, play an important role in anthocyanin degradation in various other natural and model systems (Decereau *et al*, 1956; Harib *et al.*, 1956; Lukton *et al.*, 1956; Markakis *et al.*, 1957; Meschter, 1953; Nebesky *et al.*, 1949; Pederson *et al.*, 1947; Sondheimer *et al.*, 1953; Tinsley *et al.*, 1960). All of these factors influence the red anthocyanin pigment of black and red raspberries (Daravingas *et al.*, 1965; Lee *et al.*, 1950; Pederson *et al.*, 1941; Pederson *et al.*, 1947; Tressler *et al.*, 1943).

Tinsley *et al.* (1960) investigated the kinetics of the thermal degradation of the anthocyanin pigments of strawberries as influenced by the presence of various sugars and sugar degradation products under nitrogen or air and found in all cases first order reactions. Lamort (1959a) also reported that the thermal destruction of anthocyanin pigments of red raspberry followed first order kinetics. However, he found that the anthocyanidins of the rasp-

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berry pigments did not follow the same pattern (Lamort, 1959b).

This work is an effort to study the kinetics of the pigment destruction and to further evaluate the importance of some factors influencing the degradation of the pigments in black raspberry juice, the total extracted pigment (primarily cyanidin-3-diglucoside, cyanidin-3-glucoside, cyanidin-3,5-diglucoside and cyanidin-3,5-rhamnoglucosido-5-glucoside), its major anthocyanin (cyanidin-3-diglucoside, hereafter referred to as C-3-di-G) and the common aglycone (cyanidin).

EXPERIMENTAL

THE PIGMENT DEGRADATION of Monger variety black raspberry juice was determined in three model systems: one containing the total purified pigment, another with the major component (C-3-di-G), and the third with cyanidin. Extraction, isolation and purification of the pigment were carried out as previously reported (Daravingas *et al.*, 1966). The major pigment was separated by means of paper chromatography using Whatman 3MM filter paper and butanol:acetic acid:water (4:1:5 v/v/v) solvent. The cyanidin used was obtained by acetic hydrolysis of the purified C-3-di-G (Daravingas *et al.*, 1966).

The kinetics of the thermal degradation of the pigments in the systems were studied under conditions resembling the cellular environment for the factor under consideration; the concentration of the pigment and of the reacting constituents, in each case, was in the same molecular proportion as found in the fresh berry juice. The reaction medium was a Sorensen's citrate buffer of pH 3.25.

The reactants were placed in a three-necked round bottom flask. The central neck was adapted vertically to a condenser connected to a $CaCl_2$ tube. One of the necks served as a gas inlet and the third was used for introduction of reactants and for sampling. A water bath was used to maintain constant temperature in the reaction flask. The entire system was kept in the dark. Shaking was applied to maintain homogeneous dispersion of the reactants.

The pigment concentration was determined by the spectrophotometric method of Lukton *et al.* (1956), modified to yield results for black raspberry pigment. The optical density of the pigments was determined at three wavelengths, 440 mμ, 700 mμ and the wavelength of maximum absorption, which ranged between 514 and 520 mμ. The reading at 700 mμ was taken as a measure of the turbidity of the solutions and was subtracted from both the 440 mμ and the maximum wavelength readings. From the following simultaneous equations, the optical density of the pigments at a given pH was calculated.

$$D^{max} = D_P^{max} + D_B^{max}$$
$$D^{440} = aD_P^{max} + bD_B^{max}$$

D_P represents optical density of the pigment
D_B represents optical density of the brown breakdown products.

Factor "a" is the ratio of the absorption of the pigment at 440 mμ to that at its maximum wavelength for a given pH. This ratio was determined for C-3-di-G and for the total pigment at all pH's used in the experiment. Factor "b" represents the ratio of the absorption at 440 mμ of the breakdown product to the absorption of the soluble breakdown product at the wavelength corresponding to the pigment's maximum absorption. Values for "a" and "b" are given in Table 1.

In these studies, the destruction of the pigment resulted in the formation of a red-brown precipitate which was insoluble in the buffer system employed. A soluble brown pigment also appeared which interfered with measuring the red color. Mathematical correction for minimizing the interference was presented earlier. When the reaction was allowed to proceed at 50°C for one month, the anthocyanin pigments were completely converted to the two brown products. These products have been associated with the browning of the desirable red anthocyanin color during processing and storage of fruits containing this natural pigment. Complete chemical characterization of these products of anthocyanin destruction has not been achieved.

The results obtained from these studies were analyzed to elucidate the kinetics of the anthocyanin breakdown. A plot of $\ln a/(a-x)$ against time was made where a = the initial concentration of the anthocyanin pigment and x = the fraction of pigment disappearing in time t. The first order rate constants were obtained from the slope of the resulting straight line and are given in Tables 2 and 3.

The first factor investigated was the hydrogen ion concentration of the system, which greatly affected the rate of thermal degradation of the pigment. In model systems of C-3-di-G (under oxygen or nitrogen at 50°C), a decrease in pH from 4.25 to 0.95 enhanced the pigment retention (Fig. 1 and Table 2). However, the degradation reaction proceeded faster at pH 0.95 than at pH 2.15. This finding is in agreement with Meschter's (1953) observation that maximum pigment retention was attained at pH 1.8 for strawberry anthocyanins. Changes in both directions from this pH value decreased the stability of the pigment.

Table 1. Factors "a" and "b" of the pigments at the corresponding pH.

Pigment factor		Wave length (mμ)[1]	pH 1.15	1.70	2.15	2.55	3.25	3.85	4.25	4.70	5.85	7.95	9.60
Total crude pigment	a	515	0.30	0.30	0.33	0.35	0.53	0.66	0.83	1.10	.....	.....	.....
	b	440	.....	4.10	.....	3.50	3.60	3.10	2.90	2.90	2.70	2.60	2.70
c-3-di-G	a	514	0.30	0.31	0.33	0.33	0.53	0.67	0.83	1.10	.....	.....	.....
	b	440	.....	4.10	.....	3.50	3.70	3.10	.....	2.90	2.70	2.60	2.70
Cyanidin	a	520	.....	.....	.....	.....	0.56	.....	.....	.....	.....	.....	.....
	b	440	.....	.....	.....	.....	3.20	.....	.....	.....	.....	.....	.....

[1] Wavelengths given from citrate buffer.

Table 2. First order rate constants ($days^{-1}$) for the degradation of C-3-di-G, cyanidin, crude pigment and juice.

	pH							
	4.15		3.25		2.15		0.95	
System[1]	0[2]	N[3]	O	N	O	N	O	N
C-3-di-G	0.127	0.115	0.088	0.077	0.074	0.071	0.078	0.075
Total crude pigment	0.127	0.116	0.087	0.077	0.074	0.071	0.077	0.074
Juice	0.135	.....	0.115	0.103	0.073	0.069	.....	.....
Cyanidin	.....	.....	2.23	1.00	.....	.....	.....	.....

[1] All substrates were dissolved in 0.1*M* citrate buffer. The reactions were carried out at 50°C.
[2] O represents a model system with an oxygen atmosphere.
[3] N represents a model system with nitrogen atmosphere.

Table 3. First order rate constants ($days^{-1}$) for the degradation of C-3-di-G and crude pigment in the presence of sugars.

	Sugar	Glucose		Fructose		Xylose		Sucrose		Buffer
System[1]	Conc. (M)	0.010	0.019	0.010	0.016	0.010	0.016	0.010	0.018	0.10
C-3-di-G		0.207	0.207	0.198	0.207	0.195	0.201	0.192	0.196	0.088
Crude pigment		0.208	0.209	0.198	0.207	0.196	0.202	0.195	0.196	0.087

[1] The reactions were carried out in 0.1*M* Sorensen's citrate buffer of pH 3.25 at 50°C.

The explanation of this phenomenon was that the concentration of the more stable cation form of the pigment decreased at higher pH values giving rise to the more labile nonionic form. At pH values less than 1.8, the overall stability was decreased because of the hydrolysis of the anthocyanin to sugar and the highly unstable anthocyanidin moiety. Hence, anthocyanidin degradation was able to mask the expected increased stability which would result from the higher concentration of the more stable cation form of the unhydrolyzed pigment. The initial lag period of higher stability observed at pH 0.95, probably corresponding to the time required for hydrolysis of the anthocyanin, supported the above explanation (Figs. 1 and 2).

Similar experiments using the total crude pigment in the system yielded results nearly identical to those with C-3-di-G, which was expected since the C-3-di-G is the fraction of highest concentration in the black raspberry pigment (Daravingas *et al.*, 1966) and thus would control the overall thermal degradation of the pigment (Table 2).

Additional experiments using freshly extracted juice at various pH levels (adjusted with Sorensen's citrate buffer) indicated that lowering the pH from 4.15 to 2.15 increased the stability of the anthocyanin pigment in the juice.

The higher stability of black raspberry anthocyanins in a model system as compared with the natural juice suggested that some cellular constituents were influential in this pigment destruction (Table 2). These data revealed that cyanidin was much more unstable than C-3-di-G and the natural combination of the black raspberry anthocyanins in either model systems or juice. This demonstrated that blocking of the hydroxyl group of the 3-position with a sugar retarded destruction of the desirable red pigment.

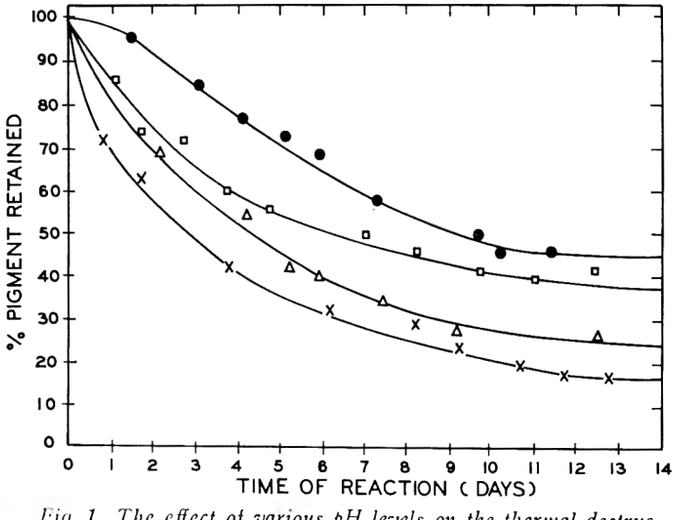

Fig. 1. The effect of various pH levels on the thermal destruction of C-3-di-G in citrate buffer (0.1M) under oxygen at 50°C. Legend: Pigment retention; ●, pH 0.95; □, pH 2.15; △, pH 3.25; and ×, pH 4.15.

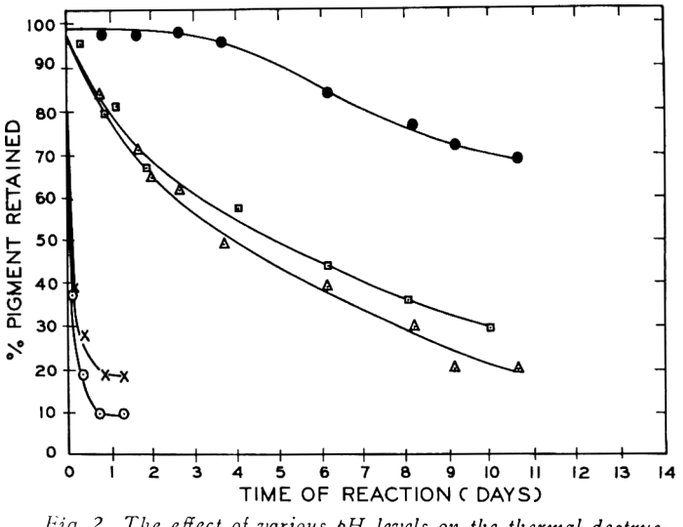

Fig. 2. The effect of various pH levels on the thermal destruction of anthocyanins of black raspberry juice and of anthocyanidin in citrate buffer (0.1M) at 50°C. Legend: Juice pigment under oxygen; ●, pH 2.15; □, pH 3.25; △, pH 4.15. Anthocyanidin; ○, pH 3.25 under oxygen; and ×, pH 3.25 under nitrogen.

A second very important factor of pigment destruction was the presence of molecular oxygen. In all systems studied, when nitrogen was used instead of oxygen, the stability of the anthocyanin or anthocyanidin was increased (Table 2). The degradation followed first order kinetics.

A third factor investigated was the effect of sugars and their degradation products on anthocyanin destruction. All four carbohydrates used (sucrose, a disaccharide; fructose, a ketohexose; glucose, an aldohexose; and xylose, an aldopentose) resulted in an increased rate of pigment degradation in model systems at pH 3.25 (the natural juice pH) and 50°C with no major differences among the sugars in this effect.

This work did not reveal the difference reported by Tinsley *et al.* (1960) that anthocyanin degradation in the presence of fructose proceeded faster than in the presence of glucose. However, their experimental conditions differed greatly from those used in this study; they used a higher reaction temperature (90°C), a higher concentration of sugars (0.25 and 0.05*M*) and a different anthocyanin (pelargonidin-3-glucoside). These conditions would markedly accelerate the reaction rate, which may account for the detection of the differences between the effects of these two sugars.

Determination of the first order rate constants by the plotting technique (Table 3) indicated that in systems with added sugars, pigment degradation was significantly faster than in corresponding systems without sugar at the same pH level. The system with added sucrose followed first order kinetics only after an initial lag period (approximately 12 hr), which was probably the time required for the hydrolysis of the disaccharide to the active glucose and fructose.

The destructive effects of the sugars may have resulted from a direct reaction of the pigment with the sugar or the pigment with some active degradation product of the sugar. Heterocyclic aldehydes may be formed under the acidic conditions prevailing in most fruit products coincident with the increased temperature of processing and may result in degradation of the pigment. Furfural can be derived from pentoses and 5-hydroxymethylfurfural from hexoses. Compounds of this type could also be derived from Maillard browning reactions or oxidation of ascorbic acid. The effect of these two compounds on the rate of anthocyanin destruction was investigated.

In a model system using C-3-di-G, both furfural and 5-hydroxymethylfurfural greatly accelerated the pigment destruction (Fig. 3). This was in agreement with the results of Meschter (1953) and Tinsley *et al.* (1960) in which both of these degradation products accelerated the anthocyanin breakdown in strawberries. Tinsley *et al.* (1960) also reported that the degradation of pelargonidin-3-glucoside followed first order kinetics in model systems of pH 3.40 in 0.05*M* but not in 0.01*M* concentrations of furfural and 5-hydroxymethylfurfural. However, in agreement with Meschter's (1953) findings, this work revealed that the reaction in the presence of the sugar degradation products did not follow first order kinetics. The reaction rate was affected by the concentration of both the anthocyanin and the added constituents, with furfural resulting in a higher destruction rate than 5-hydroxymethylfurfural.

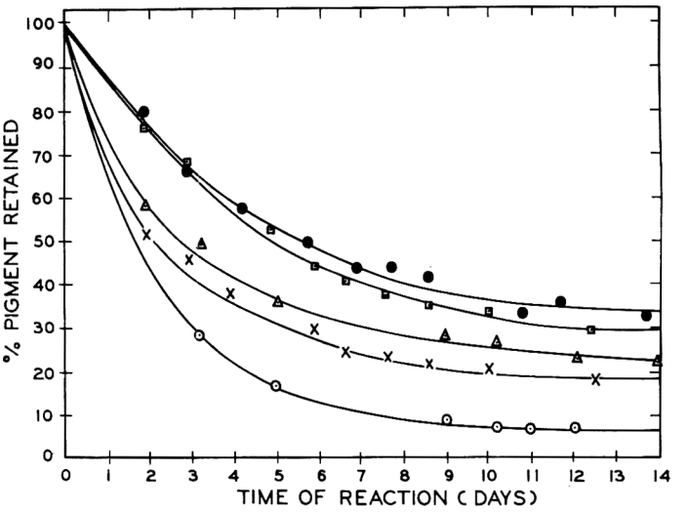

*Fig. 3. The effect of glucose and sugar degradation products on the thermal destruction of C-3-di-G in citrate buffer (0.1*M*) pH 3.25 under oxygen at 50°C. Legend: In presence of: •, 0.1*M *glucose; □, 0.1*M *formic acid; △, 0.1*M *levulinic acid; ×, 0.1*M *5-hydroxymethylfurfural; and ○, 0.1*M *furfural.*

In acidic media, the natural state for nearly all anthocyanin-containing fruits, 5-hydroxymethylfurfural may further decompose to levulinic and formic acids, so the effect of those two acids on the pigment destruction was of interest. Their reaction with C-3-di-G (in 0.1*M* citrate buffer at pH 3.25 and 50°C) revealed that pigment destruction was greater than that observed in the buffer system alone and in the presence of sugars; however, it was not as great as was observed in the presence of an equivalent concentration of furfural or 5-hydroxymethylfurfural. The breakdown of the unstable 5-hydroxymethylfurfural to the less active acids could account for the observed differences in the action of the two furanic aldehydes on the pigment.

Nearly identical results were obtained in systems containing total crude pigment with the sugars and sugar degradation products investigated (Table 3).

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Free Amino Acids in Ham Muscle During Successive Aging Periods and Their Relation to Flavor

SUMMARY—The free amino acids and total ninhydrin positive material (NPM) in a 1% picric acid extract from dry-cured hams were measured after six different periods of aging. Correlation coefficients were calculated between amino acid values and taste panel scores. Significant ($P < .05$) increases were observed for NPM, serine, glutamic acid, threonine, leucine and isoleucine (not separated), valine, phenylalanine, proline, tyrosine, alanine, glycine and histidine during successive aging periods. Correlation coefficients between NPM and the organoleptic measurements of aged flavor, acidity, elasticity, crumbliness and softness were all highly significant. It is postulated that the increase in free amino acids can be attributed to action of the naturally occurring cathepsins. The free amino acids and their changes in concentration in relationship to flavor are discussed.

INTRODUCTION

COMMERCIAL PROCESSING of dry-cured hams has increased significantly in recent years and now contributes materially to the agricultural economy of several southeastern states. This increase has been due, in part, to improved processing methods as a result of considerable product research. For example, studies of changes in certain chemical and physical properties during processing as they relate to characteristics of the final product have been cited by Craig *et al.* (1964), Ockerman *et al.* (1964) and Kelly (1965). Application of controlled aging conditions has resulted in an improvement of product (Hunt *et al.*, 1939; Fields *et al.*, 1955; Blumer, 1958; Skelly *et al.*, 1964).

Volatile compounds are considered important in the make-up of flavor. Ockerman *et al.* (1964) reported carbonyl compounds, volatile fatty acids, sulfur compounds and bases released when dry-cured ham was heated in a vacuum distillation system. The characteristic meat-like flavor originates from the lean on heating (Crocker, 1948; Pippen *et al.*, 1954; Batzer *et al.*, 1960, 1962; Hornstein *et al.*, 1963a,b; Wasserman *et al.*, 1965).

It has been suggested that certain amino acids are important precursors of the flavor components released when foods are heated and several studies have been conducted to determine the free amino acids present in different foods (Wood *et al.*, 1957; Bender *et al.*, 1958, 1961; Herz *et al.*, 1960; Macy *et al.*, 1964; Casey *et al.* 1965; El 'Ode *et al.*, 1966; Pinto *et al.*, 1966).

Muscle tissues contain active catheptic enzymes; these enzymes have not as yet been well characterized. Zender *et al.* (1958) and Sharp (1963) have shown that autolysis occurs during aseptic storage of muscle at ambient temperatures. Parrish *et al.* (1966) studied some of the properties of a purified fraction of porcine muscle cathepsin.

Because of the evidence relating amino acids to flavor, this study was undertaken to determine the quantitative changes in free amino acids of dry-cured hams during aging and to determine whether these changes were associated with organoleptic characteristics of the ham.

EXPERIMENTAL PROCEDURE

Curing and aging hams

Fifty paired skinned hams weighing approximately 6.8 kg (15 lb) each were obtained in three separate groups; two groups of 10 hams each and one group of 30 hams, with each group treated as a separate experiment. The hams were cured with 1.08 oz of a curing mixture consisting of 8 lb sodium chloride, 2 lb white sucrose and 3 oz potassium nitrate per pound of ham. One-third of the total curing mixture required for each ham was applied

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on each of the first, third and tenth days of curing and the hams were cured at 4°C for 2 days/lb.

After curing, the hams were placed in stockinettes and hung in a curing room for an additional 30 days to effect salt equalization. Thereafter, the hams were smoked at approximately 30°C by burning hardwood sawdust. They were then aged in a room at 29°C ± 3, relative humidity 60% ± 3, and air flow 35 ft/min.

Statistical design

Randomized complete block designs with two treatments and five blocks were used for the two groups of ten hams, with each pair constituting a block. Groups 1 and 2 were assigned to treatments of 20 and 36 weeks and 12 and 28 weeks of aging respectively. The 30 hams of group 3 were used in a balanced incomplete block design (Cochran *et al.*, 1950) with six aging period treatments of 0, 2, 4, 8, 16 and 24 weeks and 15 blocks with each pair constituting one incomplete block.

Sampling procedure

Five hams were removed from aging at each sampling period. Three ⅜-in. slices were removed from each ham approximately ¼-in. posterior and parallel to the aitch bone. The center slice was used for taste panel evaluation and the other two slices for the physical and chemical analysis. All determinations were made on the *semimembranosus* muscle.

An experienced taste panel of four members evaluated all hams on a 1 to 7 hedonic scale for saltiness, elasticity, crumbliness, softness, juiciness, aged flavor and acidity. The slices used for organoleptic evaluation were trimmed free of excess fat, placed on a broiler pan and broiled in a preheated oven for 10 min per side at 183°C

The first and third slices, trimmed free of fat and the dried outer layer, were homogenized in a Waring blender. Aliquots were then removed for moisture, fat and free amino acid analysis. Moisture and fat were determined as described in AOAC (1960).

Deproteinization

The aliquots for free amino acid determinations were deproteinized by the method of Tallon *et al.* (1954). The final protein free extract from the ham muscle was brought to a volume of 200 ml. Three 50-ml aliquots were transferred to three 125-ml Erlenmeyer flasks and immediately frozen with dry ice and acetone. The frozen sample was lyophilized, sealed and stored at −20°C until used for the amino acid analysis.

Separation of amino acids

Single flasks of the lyophilized sample were removed from the freezer and the dry powder dissolved in 10 ml of water.

The amino acids were separated using a modification of the method described by Rothman *et al.* (1962). Samples were applied in quantities of 20 to 60 μl per band on three strips of Whatman 3 MM filter paper 10 × 110 cm on a line 20 cm from the anode end in 2 cm bands, 3 cm apart and 1.5 cm from the edge of the strip. All quantitative amino acid determinations were run in quintuplicate.

At a distance 40 cm from the anode end of another strip, a quantity of sample double the amount used at 20 cm was applied. This application was made so that any strongly acidic compounds in the sample that migrated toward the anode during electrophoresis would not run off the end of the strip and could thus be detected.

The paper was dipped in a buffer solution of pH 1.8 (Rothman *et al.*, 1962) and electrophoresis was performed at 5,500 volts (55 volts/cm) under Varsol with an operating time of 95 min (electrophoresis tank and power supply—Savant Instruments, Inc., Hicksville, New York). After electrophoresis, the strips of paper were dried in a fume hood.

The paper strips were then cut lengthwise down the center of the strip so that each band of sample was centered on a 5-cm strip. One of the sample strips plus the strip spotted at 40 cm was dipped in a ninhydrin solution and hung in a fume hood until color developed. Using the sample strip as a marker, the five remaining strips were subjected to chromatography as described by Rothman *et al.* (1962) with the following exceptions: (1) the 10-cm strip of filter paper stitched on the top edge of the 5-cm strip was Whatman No. 1 filter paper; (2) the solvent system used was 40 parts tertiarybutyl alcohol, 30 parts 2-butanone, 15 parts formic acid (88%) and 15 parts water (Fink *et al.*, 1963); (3) the chromatographic development was stopped before the solvent ran off the paper (Fig. 1).

Samples were also applied on a sheet of Whatman 3 MM filter paper which had been previously dipped in 0.1*M* phosphate buffer at pH 9.0. A 10 × 15 cm strip of Whatman No. 1 filter paper was stitched to the top of the Whatman 3 MM filter paper. The papers were hung in chromatography jars and allowed to equilibrate with the solvent for 12 hr and then subjected to descending chromatography for 36 hr in a solvent containing 40 parts methanol, 40 parts 2-butanone, 12 parts 0.1*M* phosphate buffer and 8 parts water.

This system was used to separate carnosine. It was necessary to allow the solvent to run off the paper during the development in order to separate carnosine from the other basic and slow moving compounds.

Quantitation

The amino acids were eluted from the chromatograms and measured by the methods of Tigane *et al.* (1961) with

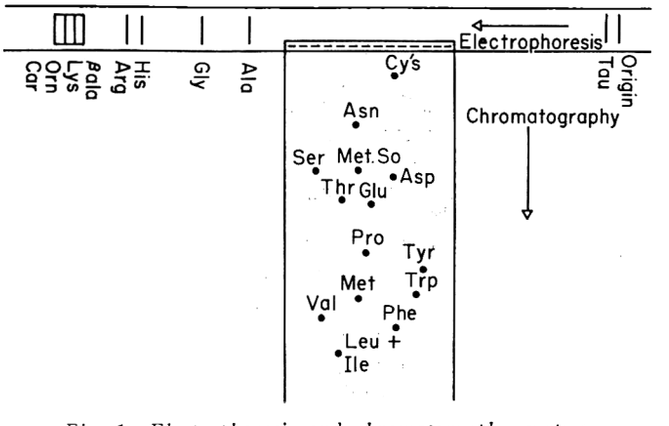

Fig. 1. Electrophoresis and chromatography system.

Table 1. NPM[1] in millimoles per gram of ham and analysis of variance for groups 1 and 2.

Weeks aged	Pairs of hams 1	2	3	4	5	Analysis of variance Source	d.f.	S.S.	M.S.	F
					Group 1					
20	0.75[2]	1.12	0.81	1.37	1.00	Blocks	4	0.415	0.107	44.4**
36	0.82	1.19	0.91	1.32	0.96	Treatments	1	0.002	0.002	0.92
Totals	1.57	2.30	1.72	2.70	1.96	Error	4	0.010	0.002	
						Total	9	0.427		
					Group 2					
12	1.12	0.92	0.64	0.72	0.72	Blocks	4	0.327	0.082	12.57*
28	1.10	1.10	0.66	0.85	0.63	Treatments	1	0.005	0.005	0.75
Totals	2.21	2.01	1.30	1.57	1.35	Error	4	0.026	0.007	
						Total	9	0.358		

[1] Total ninhydrin positive material in one percent picric acid extract.
[2] All weight values on fat-free, moisture-free basis.
* Significant ($P < .05$).
** Highly significant ($P < .01$).

one modification. It was necessary to treat the excised spots twice with the methanolic borate buffer to drive off all the ammonia from the spots. The ninhydrin-hydrindantin reagent used in the quantitation was prepared as described by Matheson *et al.* (1961). The methyoxyethanol used in his reagent was boiled with activated charcoal and redistilled out of ferrous sulfate immediately before using. All optical density readings were made at 570 mμ on a spectrophotometer (Bausch and Lomb Spectronic 20).

Total ninhydrin positive material

This determination was used as an estimate of the total free amino acids in the sample. The redissolved sample was diluted with water by a factor of 99 to 1 or greater, depending on the sample. From the diluted sample, 0.3 ml was pipetted into a screw cap test tube and 3 ml of the ninhydrin-hydrindantin reagent added. The tubes were capped, placed in boiling water for 2 min, diluted to 10 ml with 50% EtOH, rocked for 3 min and the optical density read at 570 mμ. The moles of ninhydrin positive material (NPM) were then calculated from a standard curve prepared from alanine.

RESULTS AND DISCUSSION

For groups 1 and 2, only the NPM was measured. No significant ($P > .05$) change in NPM was found to occur between 20 to 36 weeks of aging. However, there were significant ($P < .05$) differences among the pairs within these two groups.

In group 3, highly significant ($P < .01$) increases were shown for NPM and the following free amino acids: serine, glutamic acid, threonine, leucine and isoleucine (not separated), valine, phenylalanine and proline. Significant ($P < .05$) increases were found for tyrosine, alanine, glycine and histidine. In addition, the following amino acids were detected: methionine, tryptophan, taurine, arginine, carnosine, ornithine, lysine, phosphoserine, aspartic acid and asparagine (Table 2). Not all of these acids were detected in each ham. All identifications were based on relative rates of migration in electrophoresis and separation by paper chromatography. Amino acid standards were employed in conjunction with the samples in identification procedures.

Increases observed in this study for both percent soluble nitrogen and soluble amino nitrogen agree with those of Hunt *et al.* (1939) and Blumer (1954). However, Fields *et al.* (1955), except for one ham aged at 32.2°C for 12 months, observed no change in non-protein nitrogen in hams aged at varying temperatures. This contrary report may be due to the fact that the hams used in the study by Fields *et al.* (1955) were smoked to an internal temperature of 51.7 to 54.4°C, a temperature which is above the optimum for porcine muscle catheptic activity (Parrish *et al.*, 1966), and thus some of the proteolytic enzymes of the hams may have been inactivated.

The data suggest enzymatic degradation of protein although the source of enzymes, microbial or naturally occurring, is not known. Hunt *et al.* (1939) and Mundt *et al.* (1951) noted that spoilage and off flavors in hams are caused by growth of proteolytic organisms.

Zender *et al.* (1958) and Sharp (1963) observed proteolytic action in fresh meat stored aseptically at 37° and/or 25°C. Proteolysis under aseptic conditions would be due to naturally occurring muscle cathepsins. Parrish *et al.* (1966) found that the activity of partially purified porcine muscle cathepsin was maximum at pH 4.0 and optimum at pH 8.0 and 10.0, but the activity was greater below pH 5.0 than above pH 6.0. The available information suggests the proteolytic action observed during aging of dry-cured hams is primarily, if not entirely, due to the presence of muscle cathepsins.

All acids that showed significant ($P < .05$) changes during aging followed a similar time pattern. Increases were observed during the early stages of aging, but the amount of acid remained rather constant after 16 weeks. Tryptophan and taurine were the only acids that appeared to decrease with aging, however, they did not give significant ($P > .05$) F values.

Although many of the correlation coefficients determined in this study (Table 3) were highly significant ($P < .01$), those which represent a cause and effect relationship and those which merely represent parallel changes were not determined in this study.

Since Blumer (1954) and Kelly (1965) have shown significant ($P < .05$) changes in the free fatty acids during

Table 2. Average NPM and free amino acid content in micromoles per gram of ham and F values.

Amino acid[1]	Weeks aged 0	2	4	8	16	24	F Values
NPM [2,3]	506.0	651.1	709.8	927.6	924.1	990.0	13.75**
Serine	18.4	30.5	32.5	44.1	42.1	49.1	9.15**
Methionine	13.2	18.3	14.5	10.2	15.1	15.1	0.92
Glutamic acid	20.5	42.4	40.3	57.9	61.0	61.8	23.9 **
Threonine	9.7	21.8	24.3	31.8	39.9	39.7	13.7 **
Leucine + Isoleucine	32.9	64.2	63.9	92.2	94.2	100.4	21.7 **
Valine	15.3	32.0	36.4	53.5	56.4	60.0	24.3 **
Tryptophan	30.7	17.3	18.3	15.8	24.1	11.0	1.75
Phenylalanine	8.4	18.8	25.5	26.1	30.0	28.4	13.3 **
Proline	7.8	20.4	23.0	22.7	28.0	30.3	6.75**
Tyrosine	8.7	7.7	10.6	14.2	17.7	15.8	3.66*
Taurine	23.9	23.5	19.3	19.5	19.5	20.8	2.97
Alanine	48.3	77.8	85.0	63.7	97.5	101.3	3.86*
Glycine	26.4	40.7	45.7	49.1	46.8	55.1	5.22*
Arginine	12.8	18.8	16.1	16.6	16.6	21.7	2.46
Histidine	12.7	25.7	28.7	29.4	25.0	37.5	4.17*
Lysine + Carnosine + Ornithine	70.2	82.0	78.8	71.2	77.4	86.2	0.54
Carnosine	36.6	29.3	32.5	36.5	29.2	33.4	0.41

[1] Only those acids found in every ham are listed.
[2] Total ninhydrin positive material in one percent picric acid solution.
[3] Weight on a fat free, moisture free basis—average of five hams.
* Significant ($P < .05$).
** Highly significant ($P < .01$).

aging and Kelly (1965) found a correlation coefficient of 0.82 between acidity and percent free fatty acids, it seems doubtful that the correlation between NPM and acidity represents anything more than a parallel change.

Table 3. Correlation coefficients between NPM, amino acids and organoleptic qualities.

Amino acid(s)	Organoleptic factor	Correlation coefficients
NPM [1]	aged flavor	0.80**
NPM	acidity	0.62**
NPM	elasticity	0.66**
NPM	crumbliness	— .76**
NPM	softness	— .56**
Serine	aged flavor	0.78**
Methionine	aged flavor	0.02
Glutamic acid	aged flavor	0.86**
Threonine	aged flavor	0.83**
Leucine + Isoleucine	aged flavor	0.83**
Valine	aged flavor	0.85**
Tryptophan	aged flavor	— .38*
Phenylalanine	aged flavor	0.78**
Proline	aged flavor	0.76**
Tyrosine	aged flavor	0.57**
Taurine	aged flavor	— .02
Alanine	aged flavor	0.54**
Glycine	aged flavor	0.66**
Arginine	aged flavor	0.38*
Histidine	aged flavor	0.57**
Lysine + Carnosine + Ornithine	aged flavor	0.06
Carnosine	aged flavor	— .12

[1] Total ninhydrin positive material in 1 percent picric acid extract.
* Significant ($P < .05$).
** Highly significant ($P < .01$).

Sharp (1963) has shown that the main action of cathepsins is on the sarcoplasmic protein and that fibers are not attacked. However, with prolonged storage at ambient temperatures and above, catheptic action has a marked influence on texture. These reports suggest that some cause and effect relationship exists between the increases in NPM and the properties associated with tenderness (elasticity, crumbliness and softness) although undoubtedly other factions besides proteolytic activity affect these characteristics. However, Locker (1960) and Davey *et al.* (1966) stated that the tenderizing and proteolysis that take place during aging of beef at cooler temperatures are not related.

At present, considerable evidence indicates that amino acids are important as precursors of meat flavor. It is conceivable that the changes occurring in the free amino acids in dry-cured hams during aging are related to the flavor changes during the aging period. Ockerman *et al.* (1964) have isolated a number of volatile carbonyl compounds, bases and sulfides in an attempt to characterize the flavor of dry-cured hams. It has been well established that amino acids can give rise to various volatile compounds and that heating synthetic mixtures of sugars and amino acids gives rise to odors reminiscent of certain foods (Hertz *et al.*, 1960; Casey *et al.*, 1965; El 'Ode *et al.*, 1966; Pearson *et al.*, 1966). It would be expected that the amino acids in dry-cured hams are an important source of volatile compounds when the ham is heated.

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Browning and Associated Properties of Porcine Muscle

SUMMARY—A split-plot design was used to study browning and associated properties of LD muscles from 12 Duroc and 12 Poland China barrows. Pigs were: (1) untreated, (2) sugar-fed 1 week before slaughter, and (3) fasted 48 hr, then exercised to exhaustion before slaughter. One half of each carcass was cooled at 30°F and the other at 42°F. Loins and hams were evaluated for "quality" using the Wisconsin 5-point scale.

Muscles from Durocs had more marbling and ether extract than muscles from Poland Chinas; total moisture and Gardner reflectance values (browning) were lower in muscles from Durocs than in those from Poland Chinas. Color and firmness scores for hams were not affected by breed, but firmness scores of loins were higher ($P < 0.05$) for Durocs than for Poland Chinas. Glycogen, pH and reducing sugar values were similar for both breeds.

Antemortem treatment had no significant effect on marbling. Usually muscles of sugar-fed pigs had the lowest; untreated pigs, intermediate; and exercised pigs the highest color and firmness scores. Glycogen was higher ($P < 0.05$) in muscles from sugar-fed animals than in muscles from untreated or exercised animals. Muscles of sugar-fed pigs had the highest reducing sugar and lowest pH and reflectance values; those of exercised pigs had the lowest reducing sugar and the highest pH and reflectance values. Untreated pigs had intermediate values for those factors.

Muscles chilled at 42°F had lower firmness, color, and marbling scores and higher reducing sugar values than those chilled at 30°F. Simple correlation coefficients indicated relationships between reflectance values and total moisture, pH, ether extract, and reducing sugar; whereas, partial correlation coefficients indicated relationships between reflectance and ether extract and reducing sugar. As reducing sugar and/or ether extract increased, the degree of browning increased. Also, regression analyses indicated that ether extract and reducing sugar were the important factors affecting browning.

INTRODUCTION

BROWNING OF PORK is an important factor in acceptability of cooked pork. Variation (attributable to animal difference) in the degree of brownness obtained when fresh pork was heated was observed in this laboratory. Researchers at Michigan State University (Pearson *et al.*, 1962) established a positive relationship between the amount of reducing sugar present in pork tissue and the optical density of dried pork slurry (used as a measure of brownness). This relationship suggests that sugar may be partly responsible for the brown color developed upon heating.

Wilson (1965) found no difference in the brownness of low and average quality pork as defined by the Wisconsin 5-point scale (University of Wisconsin, 1963), but did find that pH was related to brownness. Thus, there may be relationships among pH, reducing sugar content, and degree of brownness obtained when fresh pork is heated.

Several reviews (Hodge, 1953; Ellis, 1959; and Reynolds, 1963 and 1965) have been published on the browning reaction in food. Although the undesirable effects of the browning reaction have been emphasized, this reaction also may produce desirable color and flavor.

The occurrence of pale, soft, exudative (PSE) pork has increased over the past few years. Factors related to the development of PSE muscles and dry, firm, dark (DFD) muscles have been established (Briskey, 1964), but the browning and related properties of those types of muscles have not been studied. The purpose of this study was to determine the effect of (1) breed, (2) antemortem treatment (untreated, sugar-feeding, and exhaustive exercise before slaughter), and (3) carcasses' chilling temperature on the development of brown color upon heating and certain properties of unheated porcine longissimus dorsi (LD) muscle. Also, the relationship of browning to those properties was studied.

EXPERIMENTAL PROCEDURE

TWENTY-FOUR (12 Poland China and 12 Duroc) barrows of similar weight were obtained from the Kansas State University swine herd and treated both ante- and post-mortem to develop three types of pork musculature, "normal," PSE, and DFD. Procedures used were based on published work related to factors that affect the development of those types of musculature in pork carcasses.

Three antemortem treatments with eight barrows (four Poland China and four Duroc) in each treatment group were: (1) untreated, to provide "normal" muscle; (2) sugar-fed, to develop PSE muscle; and (3) exercised, to develop DFD muscle. Pigs in the second group were fed a basal ration until seven days before slaughter, then fed a ration of equal parts, by weight, of sucrose and a basal ration. Pigs in the third group were fed a basal ration, fasted for 48 hr before slaughter, and subjected to exhaustive exercise immediately before slaughter.

Post-mortem treatment consisted of two chilling temperatures. After animals were stunned, exsanguinated, dehaired and eviscerated carcasses were cut in half, and each half randomly assigned to a cooler maintained at 30 or 42°F for 24 hr.

Experimental design and analyses of data

The experimental design was a split-plot (breed and antemortem treatment, whole-plot; chilling temperature, sub-plot) with four replications of each treatment combination. Data were subjected to analysis of variance; when F-values were significant for sources of variation with more than one degree of freedom, least significant differences (LSD) at the 5% level were calculated.

Simple linear and partial correlation coefficients were

computed for Gardner color-difference meter *Rd* values vs data for: reducing sugar, pH, ether extract, and total moisture. Also, a multiple regression analysis was conducted and standard partial regression coefficients were computed to study the relationship of 24-hr pH, reducing sugar, ether extract, and total moisture to browning.

Glycogen and pH

Immediately after the animals were removed from the dehairing machine, samples for measurement of glycogen were taken with a metal coring device from both the left and right LD muscle between the 5th and 9th rib, placed in plastic bags, and frozen immediately between two blocks of dry ice. Samples were removed from the carcass within 7 to 12 min after the animals were stunned. Within 5 hr glycogen was determined by the method of Seifter *et al.* (1950) with reagent stability improved by adding 1% thiourea to the anthrone reagent as suggested by Roe (1955). Values for glycogen were computed as mg of glucose per 100 g of muscle tissue.

Glass electrodes were inserted in the LD muscle immediately after samples for glycogen were removed and duplicate pH values (initial pH) were determined with a Beckman pH meter (Model 76). The carcasses were chilled at 30 or 42°F for 24 hr, then the LD muscle from the 10th rib to the 1st lumbar vertebra was excised and the pH (24-hr pH) determined by inserting the electrodes into the center of the muscle sections.

Total moisture, ether extract, reducing sugar, and browning

The entire section of muscle was wrapped in aluminum foil (0.0015 gauge), frozen at −10°F, and held at 0°F until analyzed for total moisture, ether extract, reducing sugar, and degree of browning. Each section of muscle was ground twice in an electric food grinder. Duplicate 10-g samples were dried for 2 hr at 121°C in a C. W. Brabender semi-automatic moisture tester to measure percentage total moisture.

To determine the percentage ether extract, duplicate samples of approximately 2 g of muscle were dried, extracted with ether for 16 hr on a Goldfisch extraction apparatus, the ether evaporated, and the percentage ether extract calculated. Nelson's method (1944) was used to measure reducing sugar in duplicate samples (3 ± 0.5 g) of muscle. Glucose was used as a standard, and values for reducing sugar reported as mg of glucose per 100 g muscle.

Samples that were dried to determine the percentage total moisture were powdered with a mortar and pestle and reflectance values (*Rd*) for those samples were read on a Gardner color-difference meter and used as an indication of the browning property of the muscle. The instrument was standardized with a ceramic title having a *Rd* value of 15.53.

"Quality" evaluation

Approximately 24 hr after slaughter, the loin (10th rib) and ham (butt end) were evaluated for selected "quality" factors (marbling, firmness, and color). The Wisconsin 5-point scale (University of Wisconsin, 1963) was used for the evaluation.

RESULTS AND DISCUSSION

Breed

Differences in "quality" as indicated by color, firmness, and marbling scores were noted between Duroc and Poland China pigs. In general, muscles from Durocs received higher scores than those from Poland Chinas. However, significant differences attributable to breed were found only for firmness and marbling of the loin and marbling of ham muscles (Table 1).

Loin muscles from the Duroc sugar-fed and untreated barrows were darker ($P < 0.05$) than those from similarly treated Poland Chinas; exercised Duroc barrows had lighter ($P < 0.05$) colored loin muscles than exercised Poland China barrows. Ham muscles from Duroc and Poland China barrows were similar in color (Table 1). Sayre *et al.* (1963b) also noted differences in color of muscle attributable to breed, and reported that Poland China had lighter colored LD muscles than Chester Whites or Hampshires.

Loin muscles from Poland Chinas were less firm ($P < 0.05$) than loin muscles from Duroc barrows; ham muscles were similar in firmness for the two breeds. Both the loin and ham muscles from the Durocs exhibited a greater ($P < 0.001$) degree of marbling than those from the Poland Chinas (Table 1).

In addition to "quality" differences between breeds, certain differences in muscle composition were noted. Percentage ether extract, an objective measure of intramuscular fat, was higher ($P < 0.001$), and percentage total moisture lower ($P < 0.001$) in muscles from Durocs than in those from Poland Chinas. Marbling scores, a subjective evaluation of intramuscular fat, also indicated a greater amount of fat in the muscle of the Durocs than in that from the Poland Chinas. Glycogen, reducing sugar, and both initial and 24-hr pH values were similar for the two breeds (Table 1).

Antemortem treatment

The three antemortem treatments did not affect the degree of marbling in either the loin or ham muscles; the other "quality" factors, color and firmness, were affected by the antemortem treatments. Each antemortem treatment produced loin muscles in Poland China pigs that were significantly ($P< 0.05$) different in color from the color of muscles produced by every other antemortem treatment.

Sugar-feeding produced the lightest color and exhaustive exercise the darkest. For the Duroc breed, loin muscles chilled at 30°F were darkest ($P < 0.05$) for exercised and lightest for sugar-fed pigs; for those chilled at 42°F, sugar-fed and untreated pigs had muscles that were similar in color and lighter ($P < 0.05$) than for those that were exercised to exhaustion.

Scores for firmness were significantly ($P < 0.001$) different among the antemortem treatments with the muscles from pigs given each treatment being significantly ($P < 0.05$) different from those of pigs in each of the other treatment groups. Sugar-feeding produced the least firm muscles and exhaustive exercise the most firm (Table 1). Briskey *et al.* (1959b) and Sayre *et al.* (1963a) also found that sugar-feeding produced muscles that were relatively pale and soft and that fasting and exhaustive exer-

Table 1. Mean values for four replications for "quality" scores and measurements for each treatment combination and significance of F-values and LSD's.

Factor	Chilling temperature (°F)	Antemortem treatment: Poland China Sugar-fed	Poland China Untreated	Poland China Exercised	Duroc Sugar-fed	Duroc Untreated	Duroc Exercised	Significance of F-value and LSD: Breed	Antemortem treatment	Chilling temperature	BxATxCT
"Quality" scores [1]									LSD [2]		LSD [2]
Color											
Loin	30	2.2	2.6	3.9	3.0	3.2	3.6		***, 0.53	***	**, 0.19
	42	1.8	2.6	3.9	2.9	3.0	3.5				
Ham	30	2.0	2.6	4.4	2.6	3.1	4.0		***, 0.56	**	
	42	1.5	2.2	4.1	2.4	3.0	3.9				
Firmness											
Loin	30	2.0	2.5	3.9	2.8	3.5	4.1	*	***, 0.66	**	
	42	1.5	2.5	3.8	2.4	3.1	3.6				
Ham	30	1.9	2.8	4.2	2.2	3.1	3.9		***, 0.65	***	
	42	1.5	2.2	3.9	2.0	2.6	3.6				
Marbling											
Loin	30	1.8	1.5	2.5	3.2	3.5	3.6	***		**	
	42	1.2	1.6	2.4	3.0	3.1	3.2				
Ham	30	1.2	1.8	2.2	2.5	2.6	3.0	***		**	
	42	1.1	1.6	2.0	2.2	2.4	2.6				
Glycogen (mg glucose/100 g muscle)	....	201.43	70.52	10.60	324.96	67.22	142.28		*, 103.66		
Initial pH	....	6.41	6.65	6.53	6.48	6.60	6.66				
24-hr pH	30	5.46	5.62	6.20	5.60	5.69	5.91		***, 0.22		
	42	5.44	5.65	6.18	5.59	5.68	5.82				
Reducing sugar (mg glucose/100 g muscle)	30	239.13	129.39	41.50	203.14	136.18	95.08		***, 61.52	*	
	42	243.85	139.97	44.19	215.71	148.82	97.32				
Ether extract (%)	30	2.79	2.59	2.85	5.74	6.00	6.06	***			
	42	2.96	2.62	2.82	5.61	5.78	6.26				
Total moisture (%)	30	73.46	74.06	74.40	72.00	71.98	72.40	***			
	42	73.10	73.95	74.79	71.86	72.30	72.27				
Reflectance (*Rd*)	30	16.6	22.0	27.0	12.3	15.2	17.1	***	***, 2.97		
	42	16.0	21.6	26.6	11.2	14.2	18.1				

[1] Scoring range from 1-5, 5 indicating a very firm, dark, and highly marbled muscle.
[2] Least significant difference at the 5% level.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

cise (Briskey *et al.*, 1959a) produced muscles that were dark and firm.

Sugar-feeding and exhausting pigs immediately before slaughter did not significantly affect the ether extract or total moisture of the muscle, but glycogen, 24-hr pH, and reducing sugar values were affected by those treatments. Glycogen content of muscles from sugar-fed pigs was higher ($P < 0.05$) than that from untreated or exercised pigs. Lewis *et al.* (1961) and Sayre *et al.* (1963a) reported similar results for PM and LD muscles of sugar-fed hogs.

The glycogen value for one Duroc pig that was exercised was much higher than values for other pigs in that treatment group. If this value were not included in the group, the mean of the three other values would be 9.59 mg glucose/g muscle tissue, which corresponds to the mean value obtained for the Poland China exercised pigs. It, then would appear that sugar feeding increased the glycogen in the muscle and exhaustive exercise decreased it.

Initial pH values were similar for all antemortem treatments. At 24 hr post-mortem, pH was higher ($P < 0.05$) in muscles of exercised pigs than in those of sugar-fed or untreated pigs. Muscle glycogen at the time of death of the animal influenced the amount of lactic acid formed in the muscle upon chilling the carcass.

Reducing sugar values were higher ($P < 0.05$) for sugar-fed than for untreated pigs and higher ($P < 0.05$) for untreated than for exercised pigs. Gardner reflectance values for ground tissue dried in the Brabender moisture tester were lowest for sugar-fed pigs, and highest for exercised pigs (Table 1). This indicated that muscle tissue from sugar-fed pigs browned more ($P < 0.05$) during drying than that from untreated pigs, and muscle tissue from untreated pigs browned more ($P < 0.05$) than that from exercised pigs.

Chilling temperature

Most "quality" scores were affected by chilling temperature. Color scores for hams of both breeds and all antemortem treatments, and for loins of Poland China sugar-

fed and Duroc untreated and exercised pigs chilled at 42°F were lower ($P < 0.05$) than at 30°F. Chilling temperature did not affect loin muscle color scores of Poland China untreated and exercised and Duroc sugar-fed pigs. Firmness scores were lower for both the loin ($P < 0.01$) and ham muscles ($P < 0.001$) when chilled at 42 than at 30°F. Loin and ham muscles chilled at 42°F received lower ($P < 0.01$) marbling scores than those chilled at 32°F.

Chilling carcasses at 30°F resulted in lower ($P < 0.05$) reducing sugar values than chilling at 42°F. The rate of glycolysis may be slowed by the lower temperature; therefore, the glucose resulting from glycogen break-down may be less in muscles chilled at lower temperatures. Chilling temperature did not affect other objective measurements (Table 1).

Browning

Gardner *Rd* (reflectance) values for ground, dried muscle were used to measure the degree of browning, and were affected ($P < 0.001$) by both breed and antemortem treatment. Other factors affected ($P < 0.001$) by one of those variables were 24-hr pH, reducing sugar, ether extract, and total moisture (Table 1). Several statistical methods were employed to determine the relationship of the previously mentioned factors to reflectance (degree of browning) of dried muscle samples.

Simple linear and partial correlation coefficients were calculated for reflectance vs: 24-hr pH, reducing sugar, ether extract, and total moisture (Table 2). Simple correlation coefficients indicated that each of those factors was moderately related to reflectance values. As total moisture and pH increased, reflectance values increased; reflectance values decreased as ether extract and reducing sugar increased.

Partial correlation coefficients showed significant ($P < 0.01$) negative relationships between reflectance values and both ether extract and reducing sugar (Table 2). Evidently, the apparent simple linear relation between reflectance values and either total moisture or pH was being produced by ether extract and reducing sugar. This indicated that as the reducing sugar and/or ether extract in the muscle increased, the degree of browning increased. Pearson *et al.* (1962) reported a similar relationship between browning and reducing sugar content, but found that ether extract was not related to browning.

For further study of the relationship of moisture, pH, ether extract, and reducing sugar to degree of browning (reflectance), a multiple regression equation with Y = reflectance, X_1 = total moisture (%), X_2 = pH, X_3 = ether extract (%), and X_4 = reducing sugar (mg/100 g muscle) was obtained as follows:

$$\hat{Y} = 12.962 + 0.328X_1 - 0.916X_2 - 1.466X_3 - 0.049X_4$$

Using the modified Doolittle analysis of the factors X_1, X_2, X_3, and X_4, and arranging them in order from the highest to lowest values of their simple linear correlation with Y, it was found that using X_4 alone accounted for 41.17% of the corrected sum of squares, $\Sigma (y^2)$.

When X_1 was added to the analysis, an additional reduction of 29.17% of $\Sigma (y^2)$ was obtained. A further reduction of 6.66% in $\Sigma (y^2)$ was obtained by adding X_3. Each of those three reductions was significant ($P < 0.001$). When X_2 was added, a non-significant reduction of 0.1% in $\Sigma (y^2)$ was obtained. The 4 X factors accounted for 77.1% of the variation of Y ($R^2_{y\cdot1,2,3,4} = 0.771$), so there may be important factors contributing to browning not considered in this analysis.

To find the relative strengths of the relation between reflectance and the same four factors listed previously, standard partial regression coefficients (b′) were calculated (Table 2). From the values obtained, it appeared that ether extract and reducing sugar were more effective predictors of the degree of browning than pH and total moisture, and suggested a reduced analysis of Y using only reducing sugar and ether extract.

When X_4 (reducing sugar) and X_3 (ether etract) were used to analyze Y, the multiple regression equation was:

$$\hat{Y} = 32.36 - 0.048X_4 - 1.670X_3$$

It was found that both X_4 and X_3 contributed significantly ($P < 0.001$) to the analysis of $\Sigma (y^2)$ and that β_4 and β_3 were not equal to 0 ($t = 10.12$ and 8.44, respectively). Little was lost by omitting X_1 (moisture) and X_2 (pH) since $R^2_{y\cdot3,4} = 0.769$ as compared with $R^2_{y\cdot1,2,3,4} = 0.771$. The partial correlation coefficients when only X_3 and X_4 were used, were: $r_{y4\,3} = -0.831$ and $r_{y3\cdot4} = -0.780$ ($P < 0.001$), both of which are larger than the corresponding simple r's. All statistical treatment of the data indicated that reducing sugar and ether extract were the most important factors affecting browning. As the amount of those two factors increased, browning increased.

Table 2. Simple and partial correlation coefficients and standard partial regression coefficients between reflectance and selected factors.

	Simple *r*	Partial *r*	b′
Reflectance values vs:			
Total moisture (%)	0.60 **	0.08 ns	0.084
pH (24 hr)	0.47 **	−0.07 ns	−0.049
Ether extract (%)	−0.51 **	−0.44 **	−0.530
Reducing sugar (mg/100 g muscle)	−0.64 **	−0.73 **	−0.741

**, $P < 0.01$.

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Glycolytic Intermediates and Co-Factors in "Fast-" and "Slow-Glycolyzing" Muscles of the Pig

SUMMARY—The Longissimus dorsi muscles from Chester White, Hampshire and Poland China animals were used to establish certain differences in metabolic intermediate patterns between muscles with "fast" and "slow" rates of post-mortem glycolysis. Metabolic intermediate patterns were consistent with the concept that phosphorylase is the primary control site of post-mortem glycolysis. Adenine nucleotide levels appeared to be the primary regulatory factors for phosphorylase. The phosphofructokinase and pyruvate kinase enzymes were also involved in post-mortem glycolytic control. Levels of high-energy intermediates (adenosine triphosphate, phosphocreatine and pyridine nucleotides) were much higher in the "0" hr samples of "slow-glycolyzing" muscles than in similar samples from muscles having "fast" rates of post-mortem glycolysis. No significant differences in levels of lactate or glucose were observed among these three groups in blood samples taken either at or 24 hr prior to the time of exsanguination.

INTRODUCTION

THE RATE OF POST-MORTEM GLYCOLYSIS has been found to be extremely variable in pig muscle (Briskey, 1963). A rapid accumulation of lactic acid post-mortem at near body temperature has been shown to be associated with the development of a pale, soft, exudative condition in striated muscle (Briskey, 1964; Briskey *et al.*, 1966). Previous studies by Sayre *et al.* (1963a,b) have shown that within the strains available, Chester White and Hampshire animals seldom had muscle with rapid post-mortem changes while this abnormality occurred frequently in the available strain of Poland China animals.

Strain differences within the Poland China breed provided experimental animals for previous studies on the regulation of post-mortem glycolysis (Kastenschmidt *et al.*, 1966; Kastenschmidt, 1966). It appeared that comparative studies on post-mortem glycolysis and glycolytic intermediate levels in the longissimus dorsi muscle of Chester White, Hampshire and Poland China animals would provide useful additional information on the biochemical differences between muscles having "fast" and "slow" rates of post-mortem glycolysis.

In this study, levels of glycolytic intermediates and co-factors were determined at six post-mortem periods in "slow-glycolyzing" muscles from Hampshire and Chester White animals and compared to levels found in "fast-glycolyzing" muscles in Poland China animals at similar time periods. Levels of blood lactate and glucose were also determined on samples drawn at the time of, or 24 hr prior to, exsanguination.

Evidence indicates that "fast-glycolyzing" muscles are in a highly anaerobic state prior to or simultaneous with the removal of the first sample after death. Accelerated glycolytic rates in these muscles were apparently the result of a coordinated stimulation of the phosphorylase, phosphofructokinase (PFK) and pyruvic kinase (PK) enzymes.

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MATERIALS AND METHODS

Muscle samples

All samples were taken from the longissimus dorsi muscles of *Sus domesticus* animals of the Chester White, Hampshire and Poland China breeds. Data from six animals of each breed are included in this study. The animals were carefully handled and allowed to rest prior to exsanguination. Time post-mortem was recorded with a stop watch using the beginning of exsanguination as the initial post-mortem reference. Samples (2 cm in cross section) were removed from the right longissimus dorsi muscles beginning at the area of the 4th lumbar vertebra.

The carcasses were held at 4°C and subsequent muscle samples were obtained by discarding a 1 cm thick portion at the exposed muscle area and immediately excising 2 cm cross sections, proceeding anteriorly from the 4th lumbar vertebra. The samples were quickly trimmed of excess fat and connective tissue and then immediately frozen in liquid nitrogen. Samples were taken at "0", 15, 30, 60, 120 and 180 min. post-mortem. The "0" samples were excised and frozen within approximately 3 min from the beginning of exsanguination.

Classification of muscles

Muscles were classified as "fast-glycolyzing," or "slow-glycolyzing" on the basis of the rapidity of their post-mortem change in pH. The pH values were determined on the muscle directly as described by Kastenschmidt *et al.* (1966) or on muscle samples homogenized in 5 mm sodium iodoacetate as described by Marsh (1952); the methods gave relatively comparable results.

If the muscle pH had declined to 5.5 or below at 30 min post-mortem, it was termed a "fast-glycolyzing" muscle. If the pH was 6.0 or higher at 60 min post-mortem, it was considered a "slow-glycolyzing" muscle. Muscles from the Poland China animals used in this study had "fast" glycolytic rates while those from the other two breeds had "slow" glycolytic rates according to this classification.

Preparation of muscle extracts

Muscle samples (held at liquid nitrogen temperatures) were powdered using a Waring blendor (aluminum can) pre-cooled with liquid nitrogen as described by Borchert *et al.* (1965).

The powdered samples were immediately extracted for glycolytic intermediates in the following manner: Approximately 10 g samples of finely powdered muscle (liquid nitrogen temperature) were rapidly stirred into 10 ml of ice-cold 0.6*N* perchloric acid in 50 ml pre-weighed plastic centrifuge tubes. The tubes were reweighed and sufficient ice-cold perchloric acid solution was added to provide a ratio of tissue wt (wet) to extract volume (including muscle water) of 1 g:2 ml or 1 g:3 ml. A water content of longissimus dorsi of 0.7 ml per g was assumed on the basis of previous studies in this laboratory.

The extracts were centrifuged (15,000 × g) for 15 min at 0°C and the supernatant solutions were decanted through glass wool to remove floating material. The extracts were then carefully neutralized to the methyl orange end point with 5*M* potassium carbonate. Subsequently, the precipitated potassium perchlorate was allowed to settle out at 0°C for at least 30 min, after which the analyses for metabolic intermediates in these extracts were completed within 72 hr. The analyses for the more labile intermediates were conducted first and all duplicate determinations agreed within 10%.

Separate extracts for inorganic phosphate (P_i) were prepared in duplicate according to procedures outlined by Karpatkin *et al.* (1964).

Alkaline extracts for reduced nicotinamide-adenine dinucleotide (NADH) were also prepared in duplicate by methods outlined by Klingenberg (1963a).

Analytical methods

Analyses for glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), glucose-1-phosphate (G-1-P) and glucose were carried out in the same cuvette according to the method of Hohorst (1963a). Assays for fructose-1, 6-diphosphate (FDP), glyceraldehyde-3-phosphate (GA3P), and dihydroxyacetone phosphate (DHAP) were conducted by procedures outlined by Bücher *et al.* (1963). Procedures outlined by Czok *et al.* (1963) were used to determine 3-phosphoglyceric acid (3-PGA), 2-phosphoglyceric acid (2-PGA), phosphoenol-pyruvate (PEP), and pyruvate in the same assay. Blood and muscle lactate were determined separately by the method of Hohorst (1963b). α-glycerophosphate (α-GP) was determined in another assay by the procedure of Hohorst (1963c).

Phosphocreatine was estimated by the method of Lamprecht *et al.* (1963).

Total muscle creatine assays were conducted on the diluted, neutralized perchloric acid extracts by the method of Ennor (1953).

Nucleotides were estimated by enzymic techniques described by Lamprecht *et al.* (1963) and Adams (1963).

Nicotinamide-adenine dinucleotide (NAD) was estimated by the method of Klingenberg (1963b). Reduced nicotinamide-adenine dinucleotide was estimated by the method of Klingenberg (1963a).

Inorganic phosphate was determined by the method of Wahler *et al.* (1958) on neutralized extracts prepared as previously outlined by Karpatkin *et al.* (1964).

Glycogen was estimated by a modification of the method of Pfleiderer (1963) in which GLUCOSTAT kits were used to estimate the glucose liberated by acid hydrolysis of isolated glycogen.

Assays for blood glucose were also conducted using GLUCOSTAT kits.

Chemicals

All chemicals used were of reagent grade. Distilled deionized water was used throughout the experiments. Enzymes used in the assays were of the highest purity from C. F. Boehringer and Sons as supplied by CALBIOCHEM, Los Angeles, California. Purified glycolytic intermediates and co-factors were purchased from Sigma Chemical Company, St. Louis, Missouri. GLUCOSTAT kits were purchased from Worthington Chemical Company, Freehold, New Jersey.

RESULTS

Glycolytic intermediate levels

During the first 60 min post-mortem, the levels of G-1-P,

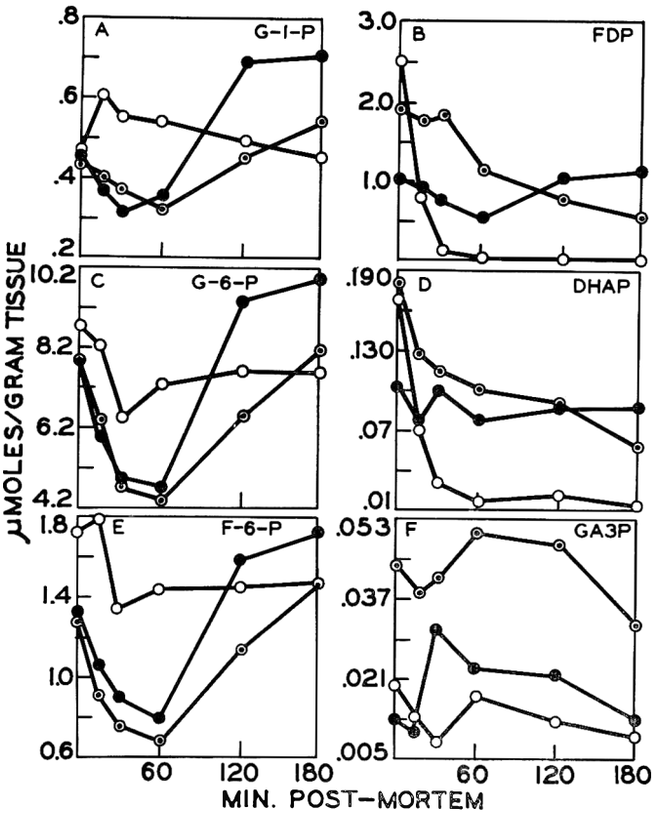

Fig. 1. Comparisons of concentrations of glycolytic intermediates in muscles from animals of the Poland China, Chester White and Hampshire breeds.

The open circles represent means from "fast-glycolyzing" Poland China muscles. The partially filled circles (⊙) represent means from "slow-glycolyzing" Chester White muscles. The closed circles represent means from "slow-glycolyzing" Hampshire muscles. Details of muscle classification are given in text.

G-6-P and F-6-P (Fig. 1A,C,E) were much lower in "slow-glycolyzing" muscles from both the Hampshire and Chester White animals than in "fast-glycolyzing" Poland China muscles.

Levels of intermediates from FDP through PEP (Fig. 1B,D,F, 3A,3C, 3E,B,D) were generally higher in "slow-glycolyzing" muscles from these breeds.

Nucleotide levels

Initial levels of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) were about 40% higher and adenosine-5′-monophosphate (AMP) levels were about 50% lower in "slow-glycolyzing" muscles from Hampshire and Chester White animals than in "fast-glycolyzing" muscles from Poland China animals (Fig. 3A,C,E).

Levels of total pyridine nucleotides were highest in Chester White muscles (Fig. 3B,D). While total pyridine nucleotide levels declined in "fast-glycolyzing" Poland China muscles during the 180 min sampling period, little decline was noted in muscles from the animals of the other two breeds.

Phosphocreatine and creatine levels

Phosphocreatine (PC) levels (Fig. 3F) were variable, but mean levels in "0" min samples were 3-fold higher in Hampshire muscles and 2-fold higher in Chester White muscles than in the "fast-glycolyzing" Poland China muscle. The post-mortem decline in PC was slowest in Chester White muscle.

Total creatine levels are shown in Table 1. The differences among the various breeds were not statistically significant ($P > .05$).

Lactate production

Fig. 2F compares the lactate levels in the musculature of the three breeds at six post-mortem periods. Lactate levels were much lower in "0" min samples of the "slow-glycolyzing" muscles from the Hampshire and Chester White animals than in similar samples from "fast-glycolyzing" muscles of the Poland China animals.

Glycogen metabolism and lactate production

Table 2 summarizes calculations on the recovery of glycogen and glycolytic intermediates as lactate. These calculations are based on means of the appropriate intermediates in the pathway from glucose and glycogen to lactic acid. In "slow-glycolyzing" muscles, the glycogen and intermediates lost during the first 60 min post-mortem could not be accounted for by lactate produced.

Glycogen, glucose and Pi levels

Glycogen levels at "0" min expressed as m*M* glucose equiv/g tissue (Table 3) were 5-fold higher in Hampshire muscles.

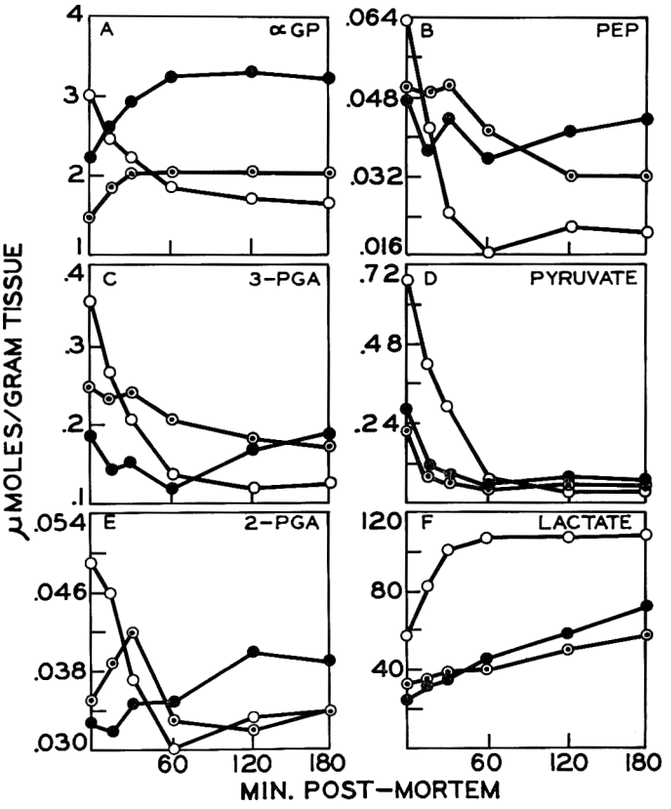

Fig. 2. Comparisons of concentrations of glycolytic intermediates in muscles from animals of the Poland China, Chester White and Hampshire breeds.

The open circles represent means from "fast-glycolyzing" Poland China muscles. The partially filled circles (⊙) represent means from "slow-glycolyzing" Chester White muscles. The closed circles represent means from "slow-glycolyzing" Hampshire muscles. Details of muscle classification are given in text.

Table 1. Mean creatine levels at death in muscles of three breeds of *Sus domesticus*.

Source of muscle[1]	Total creatine level (M/g)
Poland China	37.2 ± 0.9 (5) [1]
Hampshire	42.3 ± 1.3 (4)
Chester White	41.9 ± 0.8 (4)

[1] Poland China muscles had "fast" glycolyic rates while the other two groups had "slow" glycolyzing muscles according to the classification procedure described in text.
[2] Means are expressed ± standard error of the mean. Numbers in parentheses give number of animals studied.

Glucose concentrations (Table 3) increased with time post-mortem in all muscles studied. Glucose levels were highest in "0" min samples and also increased most rapidly post-mortem in "fast-glycolyzing" Poland China muscles.

Inorganic phosphate levels (Table 3) were all relatively low at death in the three groups of muscles. However, levels remained at less than 4 mM/g in the "slow-glycolyzing" muscles during the first 180 min post-mortem but increased to about 11 mM/g within 60 min post-mortem in the "fast-glycolyzing" Poland China muscle.

Blood glucose and lactate levels

Table 4 lists the blood glucose and lactate data for all animals represented in this study. Values for blood glucose and lactate varied widely and differences were not statistically significant ($P > .05$). Nevertheless, on the basis of ratios of blood lactate/muscle lactate there was some indication that animals with "slow-glycolyzing" muscles had more lactate in their blood at the time of exsanguination in relation to their muscle lactate than was found in animals with "fast glycolyzing" muscles (Cf. blood lactate/muscle lactate ratios in Table 4).

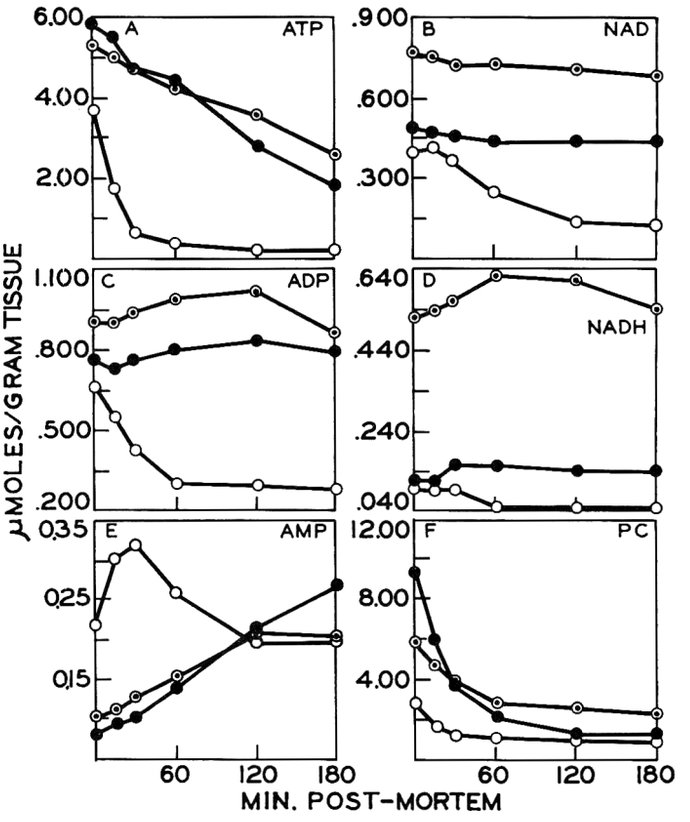

Fig. 3. Comparisons of concentrations of some high energy metabolic intermediates in muscles from animals of the Poland China, Chester White and Hampshire breeds.
The open circles represent means from "fast-glycolyzing" Poland China muscles. The partially filled circles (⊙) represent means from "slow-glycolyzing" Chester White muscles. The closed circles represent means from "slow-glycolyzing" Hampshire muscles. Details of muscle classification are given in text.

DISCUSSION

General considerations

The primary objective of these experiments was to measure glycolytic intermediate levels in muscles having "fast" and "slow" rates of post-mortem glycolysis and to correlate any observed changes in intermediate levels with differences in glycolytic rate. Since these studies were designed to answer basic questions about rapid post-mortem glycolysis, as it occurs in carcasses found in meat processing plants, the usual process of exsanguination was followed. Undoubtedly, the use of muscle relaxants and/or anesthetics would have been helpful in obtaining true resting levels of these metabolites, but it was felt that these agents would add unwarranted complications in relating

Table 2. Recovery of metabolized glycogen and glycolytic intermediates as lactate.

Muscle type	Post-mortem interval[1]	Changes during interval			Percent Recovery[5]
		Lactate[2]	Inter.[3]	Gly.[4]	
"Slow" Hamp.	"0"– 60	9.55	−2.80	— 8.45	84.8
	60–180	13.91	9.56	−22.55	104.1
"Slow" Ch. Wh.	"0"– 60	3.83	−5.38	.34	75.8
	60–180	8.84	6.75	−15.28	108.2
"Fast" Po. Ch.	"0"–180[6]	25.13	−2.43	−20.65	108.9

[1] Interval post-mortem (expressed as min).
[2] Increase in lactate during interval expressed as mM glucose equiv/g.
[3] Increase or decrease in levels of glycolytic intermediates in the pathway from glycogen or glucose to lactate, expressed as Mole glucose equiv/g.
[4] Glycogen expressed as mM glucose equiv/g.
[5] Percentages of glycogen and/or intermediates metabolites recovered as lactate and/or intermediates accumulated.
[6] Glycogen values were not determined at 60 min post-mortem in these muscles, hence percent recovery could not be calculated for the "0"–60 interval.

Table 3. Comparison of post-mortem levels of glycogen, glucose and inorganic phosphate in the longissimus dorsi muscles from three [1] breeds of *Sus domesticus*.

	Sampling time (min post-mortem)					
	"0"[3]	15	30	60	120	180
Glycogen [3]						
Poland China	23.0 ± 3.2 [4]	.....	.....	.....	.....	.8 ± .4
Hampshire	106.7 ± 4.3	.....	.....	98.3 ± 5.2	.....	75.8 ± 8.0
Chester White	35.1 ± 2.7	.....	.....	95.4 ± 2.3	.....	20.1 ± 3.4
Glucose [5]						
Poland China	3.3 ± .2	4.5 ± .1	5.3 ± .2	5.9 ± .1	6.2 ± .5	6.8 ± .3
Hampshire	1.6 ± .2	2.2 ± .2	2.3 ± .2	2.5 ± .3	4.3 ± .6	4.9 ± .4
Chester White	2.3 ± .2	2.7 ± .3	2.9 ± .3	2.9 ± .3	3.7 ± .4	4.3 ± .4
P_i [5]						
Poland China	.8 ± .5	6.5 ± 3.2	9.5 ± 4.3	11.2 ± 5.0	11.4 ± 5.4	11.3 ± 5.2
Hampshire	.2 ± .1	.4 ± .2	.4 ± .1	1.2 ± .4	.7 ± .3	.9 ± .3
Chester White	.1 ± .1	.6 ± .3	1.4 ± .7	3.9 ± .9	3.1 ± 1.0	3.9 ± 1.0

[1] Values based on six Chester White, six Hampshire and eight Poland China animals. The former two groups had "slow" glycolyzing muscles while the latter group had "fast" glycolyzing muscles as described in test.
[2] Samples were removed as the animals were exsanguinated.
[3] Glycogen expressed as *m* glucose equiv/g.
[4] Values are given as the mean ± standard error of the mean.
[5] Expressed as m*M*/g.

Table 4. Blood metabolite data from three breeds of *Sus domesticus*.

Metabolite or factor	Sample time	Metabolite levels in three breeds		
		Chester White	Hampshire	Poland China
Lactate	24 hr a.m. [1]	36.8 ± 7.0 [2,3]	47.8 ± 7.7	29.7 ± 7.3
	At death	76.1 ± 10.1	66.4 ± 11.3	102.7 ± 19.4
Glucose	24 hr a.m.	54.6 ± 3.8	54.9 ± 2.1	52.5 ± 1.7
	At death	56.5 ± 2.4	57.5 ± 4.2	56.8 ± 5.9
Blood lact. [4] / Muscle lact.		0.252	0.289	0.208

[1] Sample taken 24 hr ante-mortem.
[2] Mean value (mg/100 g blood) ± standard error of the mean.
[3] Mean values based on six Chester White, six Hampshire and eight Poland China animals. The former two groups had "slow" glycolyzing muscles while the latter group had "fast" glycolyzing muscles as described in test.
[4] Calculated from data expressed as m*M*/g tissue.

Table 5. Mass action ratios calculated from "zero" hour data on metabolic intermediates in muscles of three breeds of *Sus domesticus*.

Reaction [1]	Apparent equilibrium constant [2]	Mass action ratios in muscle of three breeds [3]		
		Chester White	Hampshire	Poland China
PGM	5.5×10^{-2}	5.7×10^{-2}	5.9×10^{-2}	5.3×10^{-2}
PGI	0.28 — .41	0.16	0.17	0.20
PFK	$1–1.2 \times 10^{3}$	0.26	0.10	0.26
ALD	$0.7–1.3 \times 10^{-4}$	0.004	0.001	0.001
TPI	0.04 — 0.05	0.24	0.14	0.12
ALD × TPI	$4–6.5 \times 10^{-6}$	1×10^{-3}	1.9×10^{-4}	1.6×10^{-4}
GAPDH × PGK	$2–15 \times 10^{2}$	1.50×10^{2}	9.77×10^{2}	3.38×10^{2}
PGlyM	0.10 — 0.18	0.14	0.18	0.14
ENOL	2.8 — 6.3	1.4	1.5	1.3
PK	$2–20 \times 10^{3}$	26.7	45.4	59.6

[1] Key to abbreviations is listed in footnote.
[2] Based on values of Minakami *et al.* (1966) and Williamson (1965).
[3] Values based on six Chester White, six Hampshire and eight Poland China animals. The former two groups had "slow" glycolyzing muscles while the latter group had "fast" glycolyzing muscles as described in text.

the results of these experiments to the problem as it usually occurs.

Throughout all of these interpretations, the assumption has been made that both "fast- and slow-glycolyzing" muscles have similar levels of all glycolytic enzymes. This is supported by the studies of Sayre *et al.* (1963a) who reported similar levels of both PFK and phosphorylase in muscles having varying rates of post-mortem glycolysis.

High energy compounds

The higher levels of ATP and PC found in "slow-glycolyzing" muscles suggest either a lower rate of ATP breakdown in these muscles or more efficient maintenance of their energy levels. From these data, it is impossible to estimate the rate of ATP turnover in the period of time immediately prior to exsanguination; yet there is no reason to expect any group would have a lower rate of ATP breakdown as all animals were allowed to rest prior to exsanguination.

When it became apparent that "slow-glycolyzing" muscles had much higher levels of ATP and PC, it was decided to study the total creatine levels in these muscles. It is known that creatine can be lost from muscle during various myopathies (Vignos *et al.*, 1963) and since the equilibrium of the creatine phosphokinase reaction is such that there is about twice as much total creatine as PC in resting muscle, a difference in total creatine would be expected to affect the level of PC. Data in Table 1 show that there was no significant difference in total creatine levels between the various groups. Therefore, at least in regard to creatine metabolism, there is ample creatine for phosphorylation and the differences in PC levels are probably the result of other factors such as available ATP for phosphocreatine synthesis.

A possible explanation for the lower ATP levels in these "fast-glycolyzing" muscles is that these muscles are in an oxygen deficient anaerobic state already prior to or simultaneous with exsanguination and that anaerobic glycolysis is unable to maintain the ATP levels. Support for this concept is presented in the next section.

Lactate metabolism

Lactate levels in "0" min samples from "slow-glycolyzing" muscles from both Hampshire and Chester White animals were much lower than those found in "fast-glycolyzing" muscles from Poland China animals. This difference cannot be due to a difference in sampling time in the initial samples because the average time from the beginning of exsanguination to freezing was 3.3, 2.6 and 2.9 min for the Chester White, Hampshire and Poland China groups, respectively.

The lower lactate levels in "0" min samples of "slow-glycolyzing" Hampshire and Chester White muscles is in agreement with results obtained with "slow-glycolyzing" Poland China muscles of a previous study (Kastenschmidt *et al.*, 1966). Since lactate levels in "0" min samples from the Hampshire and Chester White muscles in the present study were even lower than those found in "slow-glycolyzing" Poland China muscles, it appears that low "0" min muscle lactate levels are a characteristic of muscles which have "slow" rates of post-mortem glycolysis. It is likely that muscles which eventually have "fast" post-mortem glycolysis are in an oxygen deficient, anaerobic state prior to the time of exsanguination.

The higher levels of blood lactate at death tend to support this idea. Furthermore, as shown in Table 4, if one calculates the ratio of blood lactate to muscle lactate, higher ratios are observed in Chester White and Hampshire animals than in Poland China animals.

This might be interpreted to indicate more efficient removal of lactate or superior blood circulation in the Chester White and Hampshire animals immediately prior to exsanguination. Alpert (1965) studied blood lactate in connection with oxygen debt in muscle and stressed that blood lactate is not necessarily related to redox conditions within the muscle. Further studies are necessary to establish whether or not there is a relative lack of circulation in some Poland China muscles.

Results from calculations of recovery of glycogen and glycolytic intermediates as lactate reported in Table 4 also support the conclusion that "fast-glycolyzing" muscles are anaerobic already at the time of exsanguination since glycogen metabolized is recovered nearly quantitatively as lactate formed. Conversely, during the first 60 min post-mortem in the "slow-glycolyzing" muscles recovery of glycogen and intermediates metabolized as lactate was lower presumably due to metabolism of some glycogen to CO_2. This latter assumption is difficult to reconcile with the relatively low oxygen storage capacity of muscle. Further, direct studies are needed to clarify this point, although a similar observation has been made with "slow-glycolyzing" Poland China muscle (Kastenschmidt, 1966).

Possible sites of post-mortem glycolytic control

Phosphorylase. The "slow-glycolyzing" muscles from Hampshire and Chester White animals had markedly lower levels of G-1-P, G-6-P and F-6-P during the first 60 min of post-mortem glycolysis than found in the "fast-glycolyzing" Poland China muscles. These data add support to the concept that increased phosphorylase activity in "fast-glycolyzing" muscles is responsible in large part for the accelerated glycolytic rate.

Apparently adenine nucleotide levels play a large part in the regulation of phosphorylase activity in these muscles. It has been shown (Morgan *et al.*, 1964a,b,c) that both ATP and G-6-P counteract the activating influences of AMP and P_i on phosphorylase. ADP can also counteract these influences (Haugaard *et al.*, 1965). On this basis, the 40% higher ATP and ADP levels in "slow-glycolyzing" Chester White and Hampshire muscle combined with an approximate 50% lower level of AMP appear to explain the obvious decrease in phosphorylase activity and reduced rate of glycolysis in these muscles.

Inorganic phosphate levels (Table 3) remained at a lower level in these "slow-glycolyzing" muscles which would aid in controlling the phosphorylase as well as the PFK enzyme activity. Since little difference in mean "0" min P_i levels could be detected among these three groups, it seems reasonable that adenine nucleotide levels are relatively more important than P_i for control at the phosphorylase step.

In all cases in the present study, levels of the hexose monophosphates tended to decrease during the first 60 min post-mortem and to increase thereafter. This effect was

especially prominent in the "slow-glycolyzing" muscles from the Hampshire and Chester White animals even though lactate production continued at a nearly linear rate (see Fig. 2F).

This observation might be rationalized in the following manner: During initial post-mortem perods, phosphorylase *a* would be responsible for increased levels of hexose phosphates but if, as seen by Krause *et al.* (1965), phosphorylase *a* activity rapidly declines post-mortem, then production of G-1-P is not rapid enough to maintain these high levels because the activity of phosphorylase *b* is still subject to the inhibition of ATP and ADP. It is seen later that G-1-P and the other hexose phosphates increase in "slow-glycolyzing" muscles which indicates that during these periods phosphorylase activity is more than adequate to meet the demands which glycolysis would place on this enzyme.

Phosphofructokinase. It appears that some control of glycolytic flux was also exerted at the PFK step in "fast-glycolyzing" muscles when one considers the relationship between the levels of F-6-P and FDP in these muscles. F-6-P levels were higher in these "fast-glycolyzing" muscles and they also contained proportionately less FDP than did the "slow-glycolyzing" muscles, which also suggests control at this site (Cf. Fig. 1B, E). Activated phosphorylase thus appeared to increase hexose monophosphate levels in "fast-glycolyzing" muscles after which they were subjected to an apparent control by the PFK enzyme.

Indications of glycolytic control at the PFK enzyme were not, however, as clear in the case of "slow-glycolyzing" Chester White and Hampshire muscles since changes in levels of F-6-P and FDP were nearly parallel during the first 60 min post-mortem. Nevertheless, during the period 60–180 min post-mortem there was a relative increase in F-6-P compared to FDP. This latter phenomenon may be due to the decreasing muscle pH and the resultant partial inactivation of the PFK enzyme.

Pyruvic kinase. In general, levels of intermediates in the glycolytic scheme from FDP through PEP were higher in "slow-glycolyzing" Chester White and Hampshire muscles than from "fast-glycolyzing" Poland China muscles. This would suggest that control at the PK enzyme was involved in these reactions. However, during the first 15 min post-mortem, some control appeared to be exerted at the glyceraldehyde3-phosphate (GAPDH) and phosphoglycerate kinase (PGK) steps because levels of GA3P were high relative to 3-PGA and other intermediates downstream. Control at the latter site may be due to the low levels of P_i observed in these muscles. Nevertheless, control at the PK step appeared to be most important in maintaining levels of the intermediates from FDP through PEP since it can be observed that changes in PEP were roughly paralleled by changes in FDP, GA3P, DHAP, 3-PGA and 2-PGA.

It is known (Lowry *et al.*, 1964) that ATP can inhibit PK, therefore it is probable that the higher ATP levels in "slow-glycolyzing" muscles are responsible for partial control at this site. Additional support for control at the PK steps is afforded by a consideration of mass action ratios calculated from means of the appropriate intermediates in "0" min samples. Steps which operate far from equilibrium are potential control sites because there is a considerable loss of free energy.

Data in Table 5 show that the PK step operates far from equilibrium while the GAPDH × PGK, phosphoglycerate mutase (PGlyM) and enolase (ENOL) steps operate at or near equilibrium. The aldolase (ALD) and triosephosphate isomerase (TPI) steps operate somewhat displaced from equilibrium. Thus, it seems reasonable on this basis that the PK step is important in regulating levels of the intermediates from FDP through PEP. Additional evidence for control at the PFK enzyme is also given by the large displacement of the PFK step from equilibrium.

Cessation of post-mortem glycolysis. As shown in Figs. 1 and 2, lactate production ceased in "fast-glycolyzing" muscles in the presence of residual glycogen (Table 3), G-1-P, G-6-P and F-6-P. Levels of intermediates subsequent to F-6-P in the glycolytic scheme were very low, suggesting that the inactivation of glycolysis post-mortem was at the PFK site and was probably the result of decreased pH. G-1-P did not accumulate in "fast-glycolyzing" muscles after 30 min post-mortem. This could indicate that phosphorylase was also inactivated by the decreasing pH (Helmreich *et al.*, 1964) although G-1-P levels were certainly adequate to support glycolysis. Therefore it is suggested that the primary inhibition of post-mortem glycolysis is at the PFK site.

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The Non-Saponifiable Constituents of Lettuce

SUMMARY—By means of thin-layer chromatography, gas-liquid chromatography and chemical analyses the following substances were identified in dried Iceberg lettuce (*Lactuca sativa* L.): ceryl alcohol, β-sitosterol, stigmasterol, campesterol and the glycosides of the latter three sterols. An unidentified substance, probably a sterol, was detected by gas-liquid chromatography. A mixture of triterpenes identified as containing β-amyrin, α-amyrin and ψtaraxasterol was also found.

INTRODUCTION

DURING THE COURSE OF EXPERIMENTS designed to determine the subcellular site of sterol biosynthesis in higher plants it became necessary to identify the sterols in the plant tissues used in our preliminary investigations (Nicholas *et al.*, 1965). The only non-saponifiable constituent previously identified in lettuce has been ceryl alcohol (Ichiba, 1937). Nichols (1963) has identified 19 lipid components in lettuce, included in which were sterols, sterol esters and sterol glycosides. The identity of the sterols, however, was not determined.

Crosby (1963) has emphasized in an excellent review how cursory our knowledge is of the lipid constituents of many foodstuffs, including lettuce. For these and other reasons a detailed analysis has been made of the non-saponifiable constituents of Iceberg lettuce, using the combined techniques of thin-layer chromatography, gas-liquid chromatography and classical chemical analyses.

MATERIALS AND METHODS

Reagents and chemicals

All solvents were A.R. grade, distilled before use, except the ethanol used in the initial extraction which was commercial grade, used as received. The alumina was Merck, acid-washed. The petroleum ether had a boiling range of 30–60°C.

Thin-layer chromatography

Glass plates 20 × 20 cm coated with 0.25 mm of Silica Gel G (Brinkmann Instrument Co., Westbury, N. Y.) were heated for 1 hr at 100–120°C before use. The developing solvent (ascending) was benzene-petroleum ether (bp 30–60°C)-ethyl acetate (75:24:1, v/v/v).

After development the plates were sprayed with 20% antimony pentachloride in chloroform and heated briefly at 100°C to intensify the spot colors. Under these conditions the C_{29} plant sterols and many neutral C_{30} pentacyclic triterpenes separate well with R_f's of approximately 0.07 and 0.13 respectively. In the following text thin-layer chromatography will be referred to as TLC.

Gas-liquid chromatography

Gas-liquid chromatography (GLC) was performed on a Barber-Colman gas chromatograph, model 5000, equipped with a hydrogen flame ionization detector. Isothermal operation was used exclusively. The separations were accomplished on a 6-ft glass column, inside diameter 5 mm, containing a packing of 3% SE-30 on 100/120 mesh Gas Chrom Q (Applied Science Laboratories, Inc.). The column was conditioned for 48 hr at 285°C with the carrier gas flowing. The carrier gas was argon, with a flow rate of 40 ml/min, inlet pressure 24 psi. The temperatures were as follows: column—225°C, detector—260°C, flash heater—290°C.

Samples were injected in benzene using a Hamilton microliter syringe equipped with a Chaney adaptor. Retention times of the sample peaks were compared with those of known material. Reference samples were obtained as follows: campesterol—kindly provided by Dr. A. Kuksis; stigmasterol—commercially purchased; β-sitosterol—obtained in this laboratory from dried peas (Baisted *et al.*, 1962).

Preparation of non-saponifiable fractions

Four hundred pounds of Iceberg lettuce were dried in a warm room ventilated with a current of air. In a typical experiment 4.5 kg of dry lettuce was obtained from 400 lb of fresh product. The dried material was ground to a coarse powder and extracted exhaustively with absolute ethanol. The extracts were concentrated by distillation at atmospheric pressure to low volume, and then adjusted to approximately 1:1 ethanol-H_2O with 15% KOH (weight per volume).

The mixture was refluxed 1 hr, cooled, and extracted thoroughly with petroleum ether. The alkaline fraction was then thoroughly extracted with ethyl ether. Extraction first with petroleum ether and then with ethyl ether has been found to eliminate emulsification frequently encountered in extracting saponified plant extracts. Both organic phases were washed with H_2O and distilled to remove solvent. The residue, containing considerable H_2O, was filtered through paper and the filtrate extracted thoroughly with ethyl ether, the latter then washed with H_2O and distilled. All petroleum ether and ethyl ether extractable material was combined and freed of traces of solvent *in vacuo*, yielding 113 g of orange waxy material.

Crude fractionation of non-saponifiable extracts on alumina

The 113 g of non-saponifiable material was dissolved in petroleum ether and slurried onto a 9.0 cm diameter column containing 2,270 g of alumina. The column was then washed with petroleum ether until no more material was eluted. This procedure required approximately 12 L of solvent. The combined eluates on distillation yielded 1.5 g of lightly colored crystalline, waxy solid. The nature of this material is currently under study.

The column was then washed with 1:1 acetone-ethyl ether until no more solid was eluted. Distillation of the eluates left 86 g of orange, waxy product called "Sterol-Triterpene" fraction. Continued washing of the column with absolute ethanol in 4 L fractions gave 12 g of dark

gummy material in Fractions 1 to 16 from which no identifiable product could be obtained. It was eventually discarded. Fractions 17 to 23 yielded 1.2 g of white solid which was examined as the "Sterol Glycoside" fraction.

RESULTS

Further examination of the "sterol-triterpene" fraction

The acetone-ethyl ether eluate (50 g), incompletely soluble in petroleum ether, was slurried in the solvent onto a 5 cm diameter column containing 1,816 g of alumina. Eight 2000 ml fractions of petroleum ether were collected. Distillation of each left traces of yellow waxy material diminishing in quantity to less than 50 mg in Fraction 8. They were not further examined. The column was then washed with 500 ml fractions of benzene. A total of 156 fractions were collected. Distillation of the benzene left residues which were examined by TLC, the results of which are given below.

Fractions 1 to 8. A total of 10.0 g of red wax was obtained, with only traces in Fractions 1, 2 and 7. At least 11 components in Fractions 1 and 8, and in 2 to 4 were indicated by TLC. Fractions 5 to 7 consisted largely of triterpenes (brown stain with $SbCl_5$). Because of the complexity of these fractions they were not further examined at this time.

Fractions 9 to 20. In these fractions 5.0 g of fragrant orange wax was obtained. TLC indicated that the major constituent, staining brown with $SbCl_5$, was a triterpene.

Fractions 21 to 30. These fractions yielded 8.3 g of yellow solid. TLC indicated a triterpene staining purple with $SbCl_5$.

Fractions 31 to 50. A total of 7.5 g of yellow solid was recovered. TLC indicated two triterpenes close in R_f, one staining purple and the other brown with $SbCl_5$.

Fractions 51 to 156. From these fractions 17.6 g of orange wax were recovered. TLC indicated a single sterol staining brown with $SbCl_5$. The total recovery from this column was 97%.

Isolation of ceryl alcohol

Fractions 9 to 20 of the chromatographed "Sterol-Triterpene" fraction after two crystallizations from acetone yielded 4.5 g of white granules, mp 78–79°C, not raised by repeated crystallizations from the same solvent; no optical rotation in $CHCl_3$. There was no melting point depression on admixture with authentic ceryl alcohol.

An acetate, mp 61–62°C was prepared in the usual manner from this material. Since these constants are identical to those of ceryl alcohol previously isolated from lettuce by Ichiba (1937), it was not further examined. The filtrates on evaporation yielded an orange gum from which no crystalline product could be obtained from acetone or ethanol. A triterpene was indicated, however, by TLC.

FURTHER EXAMINATION OF TRITERPENE FRACTIONS

Fractions from chromatography of the "Sterol-Triterpene" mixture which contained triterpenes according to TLC (Fractions 9 to 20, 21 to 30 and 31 to 50) were freed of as much ceryl alcohol as possible by crystallization from acetone. The filtrates were accordingly enriched in triterpene as indicated by TLC. Two additional passages of the filtrate material through alumina columns (containing ratios of 1:100 of product to adsorbent) failed to effect resolution of the mixtures, based on preparation of acetates and benzoates, and as measured by TLC and GLC.

All of the filtrate products were combined, giving 11 g of light-colored solid melting at 150°. This was dissolved in 50 ml of benzene and poured onto a 50 cm diameter column containing 1,830 g of alumina. Fifty-seven 50 ml fractions of benzene were collected followed by three fractions of 20 percent anhydrous ethyl ether in benzene (500 ml each), then nine fractions (500 ml each) of anhydrous ethyl ether. The column was then discarded.

Detection of α- and β-amyrin

Fractions 5 to 10, eluted with benzene only, consisted of 2.5 g of white, crystalline solid. Only negligible traces of solid were eluted in the ether benzene fractions. TLC showed the presence of a terpene having an R_f of β-amyrin (brownish-purple color with $SbCl_5$ on heating) and an additional product considerably less polar (R_f 0.41; same color as β-amyrin with $SbCl_5$). This product is believed to be a triterpene which has yet to be identified. Repeated crystallization from methylene chloride-ethanol gave needles m.p. 208–212°; yield 0.58 g; $[\alpha]_D^{20°}$ + 41.8° ($CHCl_3$).

GLC indicated the presence of β-amyrin, α-amyrin and a product subsequently identified as ψ-taraxasterol in the proportions 41.6:17.5:40.9, (retention times of 13.9, 16.1 and 19.5 min respectively). An acetate was prepared by refluxing 0.25 g in anhydrous pyridine and acetic anhydride, with processing in the usual manner. Repeated crystallization from methylene chloride-ethanol or acetone gave needles, mp. 230–235°C; $[\alpha]_D^{20°}$ + 52.0° ($CHCl_3$).

GLC indicated the presence of β-amyrin acetate (78.3%) and ψ-taraxasterol acetate (21.7%), α-amyrin acetate having been removed by crystallization of the acetylated product. No further resolution was effected by additional crystallization from a wide variety of solvents. These constants, for both free and acetylated product, are believed to be consistent with a product containing β-amyrin and taraxasterol.

Detection of γ-taraxasterol

No solid was eluted from the column (See "Further Examination of Triterpene Fractions") with 20% ethyl ether in benzene. Fraction 1 gave 0.5 g of colorless viscous oil, while fractions 2 and 3 yielded 1.0 g of white wax. The presence of a triterpene was indicated by TLC in each of these fractions, but no further examination was made at this time. Fractions 4 and 5 yielded 7.0 g of white solid in which GLC indicated the presence of 23.5% β-amyrin, 13.6% α-amyrin and 62.9% of the third product found in fractions 5 to 10 eluted with benzene.

An acetate was prepared from the 7 g of product by refluxing in anhydrous pyridine and acetic anhydride, with processing in the usual manner. Crystallization to constant melting point from acetone, acetone-ethanol and methylene chloride-ethanol gave microcrystals, mp. 235–237°; yield 5.3 g.

Anal., Calc. for $C_{32}H_{52}O_2$: C, 81.97; H, 11.19
Found: C, 82.01; H, 11.20

Table 1. Melting points and optical rotations of some triterpenes from compositae.

Compound	Free compound		Acetate	
	m.p.°	$[\alpha]_D°$	m.p.°	$[\alpha]_D°$
α-amyrin [1]	186	+ 83.5	225–226	+ 79 (benzene)
β-amyrin [1]	197–197.5	+ 88.4		
Unknown (from Iceberg lettuce)	219–220	+ 37.8	235–237	+ 51.7
ψ-taraxasterol [2]	218–219	+ 48	237–239	+ 53
Taraxasterol [3]	224.5	+ 97	256–257 (high vac.)	+ 97
Taraxerol [4]	269–271 282–283 (high vac.)	+ 3.1	296–297	+ 8.4
Taraxol [5]	360	+ 78.6	299–301 (evac. tube)	+ 93.9

All rotations unless otherwise indicated were obtained in chloroform at temperatures (not recorded) ranging from 17 to 20°.

[1] See Pollock, 1965, page 228.
[2] See Radt, 1952, page 1161.
[3] See Radt, 1952, page 1159s.
[4] See Radt, 1952, page 1190s.
[5] See Radt, 1952, page 1916s.

On GLC the product gave a large peak and a small peak having the retention time of β-amyrin acetate which represented less than 2% of the product.

Saponification of 2.5 g of the acetate by reflux in 10% KOH in ethanol yielded a white solid on processing. Upon crystallization to constant melting point from methylene chloride-ethanol, needles were obtained, mp. 219–220°C; $[\alpha]_D^{20°}$ + 37.61° ($CHCl_3$).

Anal., Calc. for $C_{30}H_{50}O$: C, 84.43; H, 11.82.
Found: C, 84.49; H, 11.79.

The compound exhibited a single peak on GLC.

Treatment of the free triterpene (0.12 g) with benzoyl chloride and anhydrous pyridine yielded a benzoate, crystallized to constant mp. from methylene chloride-ethanol, mp. 286–288°C; $[\alpha]_D^{20°}$ + 67.0° ($CHCl_3$). Simonsen *et al.* (1951) gave mp. 288–289°C; $[\alpha]_D$ + 68° (benzene) for ψ-taraxasterol benzoate.

The melting points and rotations (for free compound and their acetates) of several compounds isolated from members of the Compositae family, of which lettuce is a member, are given in Table 1. It seems certain from these data that our unknown is ψ-taraxasterol, although no authentic reference compound was available for comparison.

Although the optical rotation of our free ψ-taraxasterol is approximately 10° less than reported for this compound in the literature, we are inclined to believe that our value is correct since on GLC only a single peak was given.

EXAMINATION OF "STEROL" FRACTIONS

FRACTIONS 51 TO 156 OBTAINED by rechromatography of the original "Sterol-Triterpene" fraction (see "Further Examination of Sterol-Triterpene Fraction") were crystallized to constant melting point from acetone and acetone-methanol giving white needles, mp. 158–160°C; $[\alpha]_D^{20°}$ −35.82° ($CHCl_3$). An acetate, prepared in the usual manner from acetic anhydride and anhydrous pyridine, on crystallization to constant mp. from acetone or acetone-methanol, gave white needles, mp. 135–137°C; $[\alpha]_D^{20°}$ −35.42° ($CHCl_3$).

A benzoate was prepared in the usual manner from benzoyl chloride and anhydrous pyridine. Crystallization to constant melting point from acetone and methylene chloride-ethanol gave rectangular plates, mp. 148–149.5°C; no optical rotation in $CHCl_3$. Gas-liquid chromatography of the free compound showed peaks having the retention time of β-sitosterol (55.7%), stigmasterol (35.2%), campesterol (9.1%) and a trace of an unidentified component (Fig. 1).

Isolation of stigmasterol acetate tetrabromide

Sterol acetate (4.2 g) in 70 ml of anhydrous ethyl ether was cooled in an ice-bath, after which 90 ml of 1:5 mixture of bromine-glacial acetic acid was added with stirring, over a period of 3 hr. After standing overnight the mixture was filtered. The precipitate was washed with a small amount of ethyl ether, yielding 1 g of white solid. This was crystallized to constant melting point from chloroform-ethanol, giving 0.3 g of white crystals, mp. 207–209°C (dec.), undepressed on admixture with stigmasteryl acetate tetrabromide; $[\alpha]_D^{20°}$ −36.0° ($CHCl_3$). The melting point and optical rotation for stigmastryl acetate tetrabromide have been given as 210°C (dec.); $[\alpha]_D^{17°}$ −39.4° (Radt, 1952, p. 1901s).

Anal., Calc. for $C_{31}H_{50}O_2Br_4$: C, 48.06: H, 6.51: Br: 41.30
Found: Br, 41.00

An additional quantity (1.5 g) of acetate tetrabromide (mp. 205–206°) was recovered from the filtrates.

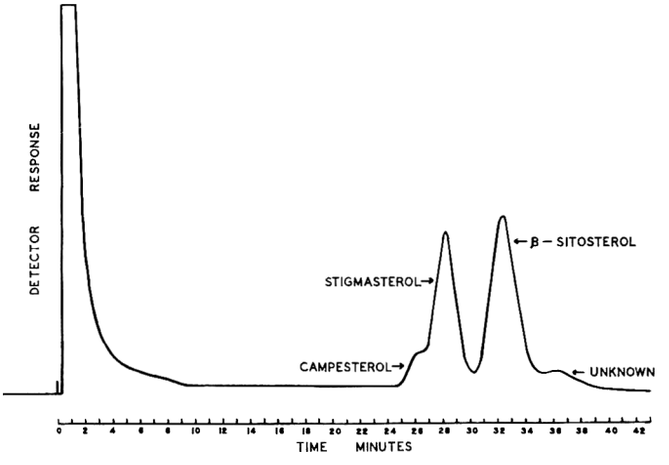

Fig. 1. Gas-liquid chromatography of the sterol mixture from Lactuca sativa *L. See Experimental Section for GLC details.*

Examination of filtrates from preparation of stigmasteryl acetate tetrabromide

After removal of stigmasteryl acetate tetrabromide the combined filtrates were freed of solvent, then debrominated according to the method of Bloch *et al.* (1958). The debrominated product was saponified with alcoholic KOH. On crystallization to constant melting point from acetone-methanol, white crystals were obtained, mp. 138–140°C. An acetate prepared from acetic anhydride and anhydrous pyridine in the usual manner gave needles, mp. 126–128°C after several crystallizations from acetone-methanol. The melting points of β-sitosterol and β-sitosteryl acetate are given as 140–141° and 126–128°C respectively (Nicholas, 1961). GLC indicated that the product was 95% β-sitosterol.

EXAMINATION OF THE "STEROL GLYCOSIDE" FRACTION

The product was crystallized to a "constant" mp. of 292–294° (dark red melt) from either a considerable excess of ethanol or benzene-ethanol mixtures, and aqueous pyridine; $[\alpha]_D^{20°}$ −30.0° (pyridine). From other batches of lettuce products mp of 282–283°C and 295–298°C were obtained. The mp of β-sitosteryl-D-glucoside has been recorded in various ranges between 285° and 305°C with optical rotations from −40 to −50° (Radt, 1952, p. 1820s).

Anal., Calc. for $C_{35}H_{60}O_6$: C, 72.87; H, 10.45 (β-sitosteryl-D-glucoside)
Found: C, 72.40; H, 10.27

Acetylation (acetic anhydride and pyridine under reflux 1 hour) and processing in the usual manner gave a product which was recrystallized from acetone to mp 168–170°C; $[\alpha]_D^{20°}$ −24.6° ($CHCl_3$).

Anal., Calc. for $C_{43}H_{68}O_{10}$:C, 69.32; H, 9.20 ("sitosterolin tetraacetate")
Found: C, 69.00; H, 9.40

Hydrolysis of sterol glycoside

The sterol glycoside (271 mg, mp 292–293°) was refluxed for 20 hr in a mixture of 10 ml concentrated HCl and 50 ml ethanol. After dilution with H_2O the mixture was extracted with ethyl ether, which was washed thoroughly with H_2O and distilled to dryness. The residue on several crystallizations from acetone gave needles, mp 135–137°C. GLC indicated the presence of β-sitosterol (55.7%), stigmasterol (35.2%) and campesterol (9.1%). Because of the high proportion of stigmasterol present the melting point of this product could probably be raised to the value found for the free sterol mixture (mp 156–158°C), but the product was not further examined.

DISCUSSION

THE RESULTS OF THE PRESENT WORK indicate why some of the earlier investigations with lettuce left some doubt about the exact nature of the non-saponifiable constituents, especially the sterols and triterpenes. The "phytosterol" of Olcott *et al.* (1931) could very well be identified as a single compound, for example, with less sophisticated instrumentation available. Since the report of Thompson *et al.* (1963), it is apparent that sterols from higher plants having melting points and optical rotations in the range formerly given for "gamma sitosterol" are mixtures of β-sitosterol and campesterol.

In the case of lettuce a similar situation was found, with β-sitosterol and stigmasterol as the major components and campesterol and an unidentified component as minor constituents. (The close association of this unidentified component with the sterols would make it likely that it is a sterol also.) Every sample of β-sitosterol isolated from plants in our laboratory has been found by GLC to contain varying proportions of stigmasterol and campesterol, even though melting points and optical rotations of the mixtures fall within the range of those given for this sterol.

Although the nature of the carbohydrate portion of the mixed sterol glycoside was not determined it seems fairly certain that it was glucose in view of the observations of Nichols (1963) that the lettuce sterol glycoside contained this sugar as the carbohydrate moiety. The ratio of sterols in the glycoside mixture closely approximates that in the free sterol mixture.

In our early experiments in which 0.5 to 1 kg quantities of dried lettuce were examined, a small quantity (less than 100 mg) of crystalline solid with a camphor-like odor sublimed from the non-saponifiable fractions on removal of last traces of solid. This solid soon evaporated at room temperature and was therefore not further examined. Perhaps due to the extensive manipulations involved in processing large quantities of lettuce, the material, very probably a terpene, was not found in subsequent operations. Some other unidentified trace non-saponifiable constituents have been indicated also.

It is believed that the complexity of the non-saponifiable fractions of lettuce may account in part for the wide distribution of C^{14} in subcellular particles after incubating minced lettuce with mevalonic acid-2-C^{14} (Nicholas *et al.*, 1965).

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Chemical Modification of Egg White with 3, 3-Dimethylglutaric Anhydride

SUMMARY—Egg white, near its normal concentration, was reacted at 25°C, pH 9.0, with levels of 3,3-dimethylglutaric anhydride (DMGA) from 0 to 60 mol/mol egg white protein (EWP) (assuming egg white to have an average mol wt of 50,000). Analysis for amino acids and functional groups showed about 26% of the lysine residues reacted at 15 mol DMGA/mol EWP. Sulfhydryls are more resistant to reaction with 60% unreacted at 150 mol DMGA/mol EWP. Electrophoresis indicates significant changes in net charges on the protein, particularly on lysozyme which migrates anodically in six separate bands at higher DMGA levels. Ultracentrifugation sedimentation data suggest no hydrolysis or aggregation changes. Foam formation is not seriously altered, but the heat coagulation properties, as evidenced by angel cake failures, are changed. As measured by viscosity, light transmission and aerating ability, DMGA exerts a protective action against the effect of heat on these properties.

INTRODUCTION

THE EGG WHITE PROTEIN SYSTEM, because of its several proteins with interesting biological properties, has interested biochemists, embryologists, and food technologists for many years. Several of the egg white proteins have been obtained in highly purified forms and their physical and chemical properties extensively studied. The egg white proteins have been comprehensively reviewed by Fevold (1951), Warner (1954), Feeney (1964), and Parkinson (1966).

Because of its ability to form a stable foam, its heat denaturation properties, its adhesive and binding characteristics, and its high nutritional quality, egg white has found many uses in the food industry. However, the pasteurization of unmodified egg white at the temperatures necessary to kill all pathogenic bacteria decreases the functional performance in angel food cakes. One of the procedures used to solve this problem is to lower the pH and form a metal complex with the ovotransferrin (Cunningham *et al.*, 1965) and to use lower temperatures in combination with hydrogen peroxide (Lloyd *et al.*, 1957).

Decreases in the whipping ability of dried egg white are restored by the addition of sodium dodecyl sulfate and a wide variety of other compounds (Bergquist, 1964). In addition to the well-known and extensive use of egg white in the bakery and confectionery trade, it has been employed in photographic applications, in wine clarification, as an antioxidant (Marion *et al.*, 1964), as a binder in sausage type products, and in the production of artificial fibers (Lundgren, 1946).

Attempts to modify the properties of egg white and its proteins can be traced to the work of Fraenkel-Conrat *et al.* (1945) using formaldehyde and to that of Neurath *et al.* (1945) and Lundgren *et al.* (1949) using synthetic detergents. Chemical modification of ovomucoid by alkylation and reduction was reported by Deutsch *et al.* (1961). Simlot *et al.* (1966) used acetic anhydride, succinic anhydride, ethyl acetimidate hydrochloride, and iodine to increase the acidic properties of ovomucoid. Buttkus *et al.* (1965) found that acetic anhydride, potassium cyanate, and succinic anhydride produced a more acidic ovotransferrin but these reagents did not greatly alter its sedimentation constant.

Many of the functional characteristics of proteins depend upon the distribution and quantity of charged groups and physical structures. Changes in charges on these proteins or in their physical structures result in modified properties. Herriott (1935) reported that the amino groups of proteins can be modified by acylation with ketene. Ketene was also shown to react with the available sulfhydryl groups of egg albumin by Fraenkel-Conrat (1944).

Mellon *et al.* (1947) reported that the amino groups of proteins could be selectively acylated with benzoyl chloride. Carbobenzoxychloride, m-chlorobenzoyl chloride, p-chlorobenzoyl chloride, and benzene sulfonyl chloride have also been widely used for the acylation of proteins. Carbon suboxide was shown to react with the sulfhydryl groups of egg albumin in addition to amino groups (Tracy *et al.*, 1942). Phenyl isocyanates were used by Kleczkowski (1960) to "modify" amino groups. Creech *et al.* (1940) demonstrated that 1,2-benzanthryl isocyanates react preferentially with amino groups. Sulfonation and phosphorylation of proteins have been used to introduce acidic groups into proteins.

Williams *et al.* (1966) found that the aldehyde groups of salicylaldehyde react with amino groups of proteins to form a reversible Schiff base. 1-fluoro-2,4-dinitrobenzene has been reacted with a wide variety of proteins. Di-nitrophenylation has been used for modification and structural investigations of proteins (Hirs *et al.*, 1965).

This report is a portion of a broad-based investigation to develop and evaluate chemical and enzymic means of altering the functional properties of egg white as a basis for developing improved or new egg products of increased value. In this report the effect of glutarinating egg white on selected functional, physical and chemical properties of the resulting product is included.

These investigations deal primarily with the chemical modification of whole egg white in concentrations near those found in the commercial product. It is anticipated that modification in such a complex system would proceed differently from that in the isolated, dilute protein solutions used in more typical biochemical studies. Chemical modification of purified proteins is carried out only where necessary to elucidate the changes observed in whole egg white.

EXPERIMENTAL

Preparation of samples

The egg white used in these experiments was prepared from laboratory broken fresh eggs procured from the Iowa State University Poultry Farm. The albumen was separated from the yolk with a breaking cup and blended (without foam formation) by placing it in a Waring blendor and flicking the switch several times to shear the ovomucin fibers. The egg white was placed in one-quart ice cream containers, frozen, and stored at −20°C. Egg white was thawed overnight at 4°C, re-blended and strained through cheese cloth prior to chemical modification.

Solid 3,3-dimethylglutaric anhydride (DMGA) was slowly added to egg white with continuous stirring. DMGA was added at levels of 3, 6, 15, 30, 60, and 150 mols of reagent per "mol" of egg white protein (assuming egg white to have an average mol wt of 50,000). The reaction was carried out at room temperature, with continuous stirring in a covered beaker for 4 hr, and then held at 3°C until used.

Dilute NaOH (0.1–1.0*N*) was added on a continuous basis to maintain the reaction pH at 9.0. Preliminary experiments indicated all reactions were essentially complete in 4 hr at 25°C.

Amino acid analyses

The amino acid analyses were performed with a Technicon Auto Analyser. The samples were hydrolyzed with 6*N* HCl in evacuated tubes for 22 hr at 110°C.

Free amino group determination

The free amino groups present in the proteins were determined by the colorimetric method using 2,4,6-trinitrobenzene sulfonic acid described by Habeeb (1966).

Sulfhydryl groups

Ellman's reagent (1959), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), a water soluble disulfide for the determination of sulfhydryls, was adapted for use with 1 ml of protein solution, buffered to pH 8.0 with 0.1*M* sodium phosphate, and added to 2 ml of deionized water. To this solution was added 5 ml of 0.96% sodium dodecyl sulfate in the phosphate buffer. An additional 2 ml of the phosphate buffer was then added to the mixture. Three ml of this solution was mixed with 0.02 ml of DTNB color reagent. The DTNB was prepared by adding 36.7 mg DTNB to 10 ml of pH 7.0, 0.1*M* sodium phosphate buffer. A 3 ml sample of solution with no added DTNB was used as a reference solution. The color of the protein-DTNB was allowed to develop for 5 min and the optical density read at 412 mμ.

Iron-binding capacity

The iron-binding capacity was measured by the method described by Fraenkel-Conrat *et al.* (1950).

Lysozyme activity

The lytic activity of lysozyme is assayed by its ability to break the cell walls of a *Micrococcus lysodeikticus* suspension. The extent of clearing, which can be measured by an increase in the transmission of the suspension, is an estimate of the lysozyme activity. The determination of the activity is based on the method of Smolelis *et al.* (1950), but improved results were obtained by using a pH 7.1 buffer, as reported by Wilcox *et al.* (1954).

Electrophoresis

Horizontal starch gel electrophoresis was performed with the discontinuous buffer of Poulik (1957). The gel buffer contained 0.076*M* tris, 0.005*M* citric acid, and 2.0*M* urea at pH 8.6. The electrode buffer contained 0.3*M* boric acid and 0.06*M* NaOH. A concentration of 12% starch was used. Electrophoresis was carried out at 200 volts and 15 ma for 16 hr. The starch gels were stained for 10 min with a solution of 1% amido black and then repeatedly washed with a washing solution of methanol, water, acetic acid, and glycerin (10:10:1:1).

Immunochemical studies

The antibody to ovotransferrin was prepared by the method outlined by Campbell *et al.* (1964). A 1% solution of ovotransferrin in saline was emulsified with 1 ml of Freund's complete adjuvant and was injected intravenously at four sites in the rabbits. The injection was repeated at 2-week intervals. After 6 weeks, the rabbits were bled from the ear and their serum tested for antibody reaction toward ovotransferrin. Upon obtaining a positive reaction, blood was removed from the rabbits by cardiac puncture and serum was separated by allowing the blood to stand at room temperature for 4 hr. The isolated serum was refrigerated overnight and centrifuged to remove contaminating cells. This serum was then frozen and stored.

One percent Noble agar (Difco Laboratories, Detroit, Michigan) was dissolved in a gel buffer consisting of 0.01*M* diethyl barbituric acid, 0.05*M* sodium diethylbarbiturate, and 0.05*M* sodium acetate, pH 8.6. This solution was warmed in boiling water, and the dissolved agar was poured evenly on a clear microscope slide, which had earlier been coated with a solution of 0.1% agar and 0.05% glycerin to improve adhesion. The gel was allowed to set for 4 hr. The gels were punched with a punch die for troughs and wells and the gel plugs removed by suction. The antigens to be tested were placed in the upper and lower wells, and electrophoresis was carried on at 250 volts, for 1 hr at room temperature, using the veronal buffer in which the gel had been dissolved.

Immediately following electrophoresis, the gel from the trough was removed and the serum containing the antibody was added. The slides were then left in a humid chamber at room temperature for 20 hr. When the precipitation lines had clearly developed, the plates were washed with saline to remove the unprecipitated protein, stained with amido black, and washed with the same solution as that used on the starch gel plates.

Ultracentrifugal analysis

Analytical ultracentrifugal analyses were made with a Beckman Model E analytical ultracentrifuge. Aluminum, 4°, 12 mm, single sector centerpieces were used in an AN-D rotor at a rotor speed of 59,780 rpm. Schlieren optics were used, and the temperature was maintained at 20°C. Tris buffer with a pH of 8.00 and an ionic strength of 0.10 were used. Protein concentrations of 0.25, 0.50, 0.75, 1.00, and 1.20 were run and the results extrapolated

to zero concentration. S values are reported as equivalent values at 20°C and in water.

Angel food cake performance

The angel food cake performance of egg white depends on the whipping ability, foam stability, and heat denaturation properties of the egg white proteins. Changes in physicochemical properties of egg white proteins can be ascertained by baking cakes containing modified egg white and evaluating differences in them. The procedure used here was similar to that described by MacDonnell *et al.* (1950). The ingredients for the cakes consisted of 61 g of egg white, 62.5 g of sugar, 22.5 g of flour (sifted four times), 0.9 g of cream of tartar, and 0.3 g of sodium chloride. The egg white was beaten at 25°C in a Hobart Kitchen-Aid K5-A mixer with a wire beater. After 10 seconds of beating, the cream of tartar and salt were added. One-fifth of the sugar was added after 20, 30, 37, and 45 sec.

After the estimated optimum beating time (by visual observation) the specific gravity of the meringue was determined. Then the remaining one-fifth of sugar, sifted with all the flour, was folded in, one-fourth at a time, using 10 strokes after each addition. The specific gravity of the batter was determined, and 120 g of batter were placed in a 2¾ in. × 3½ in. × 5½ in. pan and baked at 350°F for 27 min. The cakes were cooled and their volume measured by rape seed displacement. The crumb pH was recorded.

Emulsifying capacity

The emulsifying capacity of the proteins was determined by a modification of the method of Pearson *et al.* (1965). The method essentially consisted of making up solutions containing 6.0 mg of protein nitrogen in 10 ml of 0.6*M* KCl buffered to the desired pH with 0.01*M* buffer. Cottonseed oil was added at the rate of 0.6 ml/sec while stirring at a speed of 1,500 rpm until the emulsion broke. The emulsifying capacity is expressed as g of oil emulsified/mg of protein nitrogen.

Heat stability

The pH of the reacted egg white was lowered to 8.5 by very slowly adding 0.5*N* HCl in saline. The concentration of the proteins was then reduced to 16 mg of nitrogen per ml by diluting with pH 8.5, 0.15*M* tris buffer.

Heat treatment was carried out on 250 ml samples of the diluted egg white in 400 ml Berzelius beakers. Heat treatments were accomplished by first preheating all the samples to 50°C in a water bath. The preheated samples were then heated in a 60°C water bath for 0, 3, 5, 10 and 15 min and immediately cooled to 25°C in an ice bath. A separate control (identified as 00) was not preheated, but was held at room temperature.

Relative viscosity of heated glutarinated egg white

The viscosity of the solutions at 25°C was determined with a Cannon-Fenske size 150 capillary viscometer and is reported as relative outflow time of samples to water at 25°C.

Transmission of heated glutarinated egg white

Transmittancy measurements (Seideman *et al.*, 1963) were made at 550 mμ on a Bausch and Lomb Spectronic 20. One hundred percent transmittancy was set with distilled water.

Relative specific gravity of the meringue of heated glutarinated egg white

The relative foaming ability of the glutarinated egg white after heating was determined by using 61 ml of the diluted egg white with 47 g sugar, 0.9 g cream of tartar, and 0.3 g sodium chloride. The whipping was carried out and the ingredients were added as described earlier for forming the meringue for the angel food cakes, except that this mixture was beaten for 1, 2 and 3 min. The specific gravity of the meringue was determined by dividing a weighed amount of the meringue by its volume.

RESULTS AND DISCUSSION

Amino acid analyses

The amino acid composition of unreacted and glutarinated egg white and unreacted and glutarinated ovalbumin is shown in Table 1. When 15 mols 3,3-dimethylglutaric anhydride/mol egg white protein were reacted at 25°C for 4 hr, lysine appeared to be the only amino acid significantly affected. Approximately 11 residues of lysine per "molecule" react under these conditions. In ovalbumin, under similar reaction conditions, approximately eight residues of lysine per molecule reacted.

Table 1. Amino acid composition of native and glutarinated egg white and ovalbumin ("mol wt" egg white = 50,000).

Amino acid	Egg white	15 mol DMGA / mol egg white	Ovalbumin	15 mol DMGA / mol ovalbumin
Lysine	28	17	21	13
Histidine	9	9	7	7
Arginine	16	16	15	15
Aspartic acid	43	44	31	32
Threonine	23	23	13	14
Serine	41	41	27	27
Glutamic acid	42	41	49	48
Proline	15	15	14	14
Glycine	51	51	19	19
Alanine	61	60	36	37
Cysteine	4.5	4.4	5	5
Valine	34	33	32	31
Methionine	12	12	16	16
Isoleucine	20	20	24	25
Leucine	35	34	32	31
Tyrosine	7	7	10	10
Phenylalanine	15	15	20	20

Table 2. Changes in free amino and sulfhydryl groups in glutarinated egg white.

Mol DMGA/mol EWP	Relative free amino groups[1]	Relative sulfhydryl groups[2]
0	100	100
3	95	....
6	75	....
15	47.5	....
30	37.5	90
60	20	75
150	10	60

[1] Original free amino groups = 28 residues/mol "egg white."
[2] Original sulfhydryl groups = 4.5 residues/mol "egg white."

Table 3. Iron-binding capacity of glutarinated egg white.

Mol DMGA/mol EWP	Relative iron-binding capacity
0	100
1	111.3
3	101.0
6	79.7
15	41.6
30	35.9

Changes in sulfhydryl and free amino groups

Table 2 shows that DMGA rapidly reacts with the free amino groups in egg white. Glutarination also tends to reduce the "free" sulfhydryl groups in egg white although the rate of sulfhydryl reduction is less rapid than the rate of reaction with free amino groups. This would be expected since the number of potentially available sulfhydryls is only 4–5 and some molecular rearrangement may be required to permit their reaction.

Iron-binding capacity

Glutarination of egg white with low levels of DMGA may slightly increase the iron-binding capacity of the egg white, as indicated in Table 3. At levels above 6 mol DMGA/mol EWP a significant decrease in the iron-binding capacity of ovotransferrin occurs. This would be predicted on the basis of the involvement of amino and tysrosyl groups in the metal-binding reaction and the blocking of these groups with DMGA. Free amino group reduction, as indicated in Tables 1 and 2, is closely related to the reduction in iron-binding capacity, confirming the mechanisms reviewed by Feeney *et al.* (1966).

Lysozyme activity

The lysozyme activity of glutarinated egg white is rapidly reduced (Table 4). In view of the marked changes in electrophoretic patterns of lysozyme at low levels of glutarination, this would be expected. Lysozyme, with its large proportion of basic groups (I.P. = 10.5), should be especially susceptible to glutarination.

Table 4. Lysozyme activity of glutarinated egg white.

Mol DMGA/mol EWP	Mg lysozyme activity, ml egg white
0	3.60
6	1.90
15	0.55
30	0.019
60	0.002

The addition of a large number of glutaryl groups would alter the interaction of the lysozyme-ovomucin complex, the heat stability properties of the protein, and its amphoteric properties as well as its lytic ability.

Electrophoresis

The electrophoretic behavior of glutarinated egg white is shown in Fig. 1. The major observable change in the electrophoretic patterns is in the increased anionic migration of all the proteins. This change in mobility would be expected upon the addition of glutaryl groups. In the lysozyme region, the presence of more than one band is indicative of a major change in the lysozyme molecule. Fig. 2 shows that the electrophoretic patterns of isolated lysozyme reacted with various amounts of DMGA, confirming the observations in whole egg white.

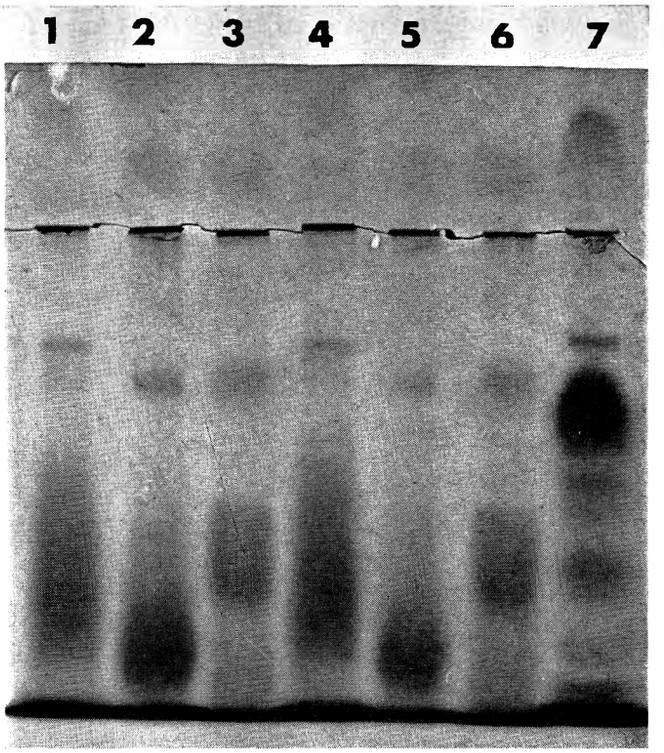

Fig. 1. Starch-gel electrophoretic patterns of glutarinated egg white. (1, 2, 3) 60, 30 and 15 mols DMGA/mol EWP, respectively, reacted for 8 hr at 25°C; (4, 5, 6) 60, 30 and 15 mols DMGA/mol EWP, respectively, reacted for 4 hr at 25°C; (7) control egg white.

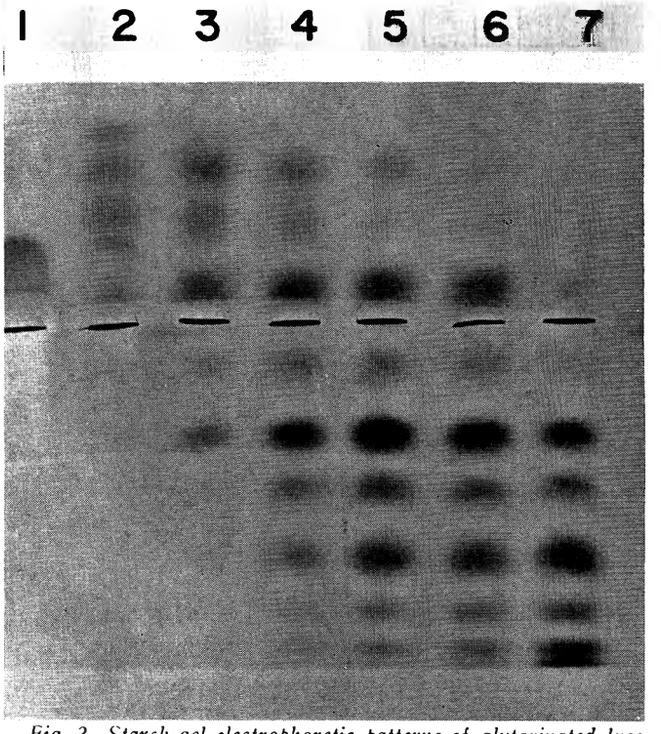

Fig. 2. Starch-gel electrophoretic patterns of glutarinated lysozyme. (1) Control lysozyme; (2, 3, 4, 5, 6, 7) lysozyme reacted with 2, 4, 8, 10, 12 and 30 mols/mol lysozyme for 4 hr at 25°C.

No satisfactory explanation can be presented for the increased cationic migration of lysozyme at low levels of DMGA or for the anodic separations into six distinct bands at higher levels. The increased anionic migration with increased levels of DMGA is of course due to the blocking or replacement of basic groups with the carboxyl of DMGA and the resulting change in net charge. Other functional groups with an active hydrogen may also be involved. The isolation and further characterization of the individual derivatives may offer an explanation in the future.

Immunoelectrophoresis

The results of the immunoelectrophoretic studies are shown in Fig. 3. On each slide the lower well contains egg white reacted with 15 mols DMGA/mol EWP; in Slide 2 the well contains egg white reacted with 30 mols of DMGA; and on Slide 3 it contains egg white reacted with 60 mols of DMGA. The location of the precipitation lines indicates that glutarination changes the mobility of the protein but that immunochemically the protein antigen is still capable of combining with the antibody. It is concluded that glutarination does not seriously affect the antigenic sites of ovotransferrin.

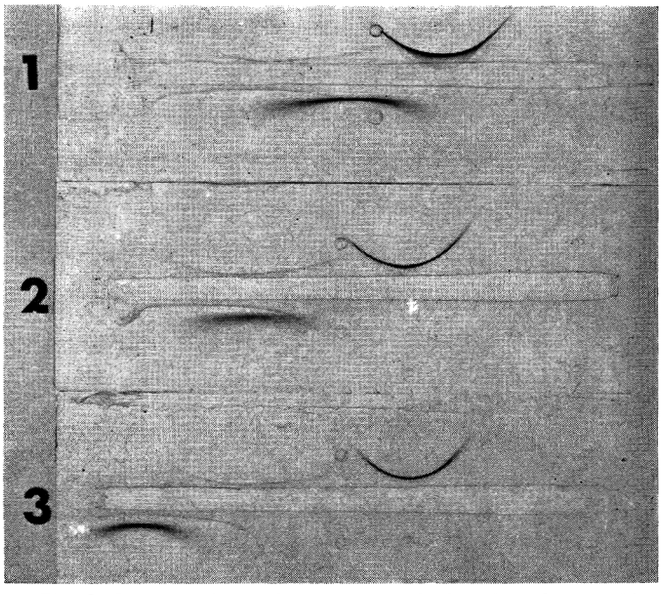

Fig. 3. Immuno-electrophoretograms of glutarinated egg white. Upper well contains egg white control in 1, 2 and 3. Lower well contains egg white reacted with 15, 30 and 60 mols DMGA/mol EWP in 1, 2 and 3, respectively. Center trough contains conalbumin antibody in 1, 2 and 3.

Ultracentrifugal analyses

Despite the fact that egg white contains several proteins, upon analytical ultracentrifugation, it sediments as one moderately diffusing peak and one minor slightly faster peak (Fig. 4). The $S_{20,w}$ for the major peak extrapolated to zero concentration is 2.80, and the $S_{20,w}$ for egg white

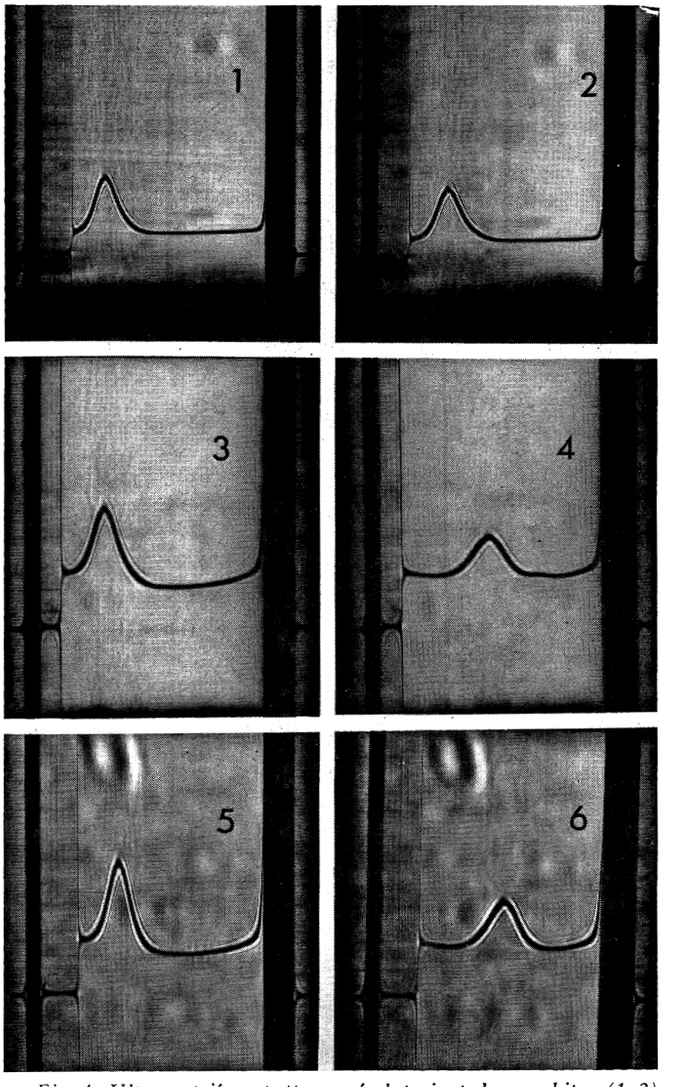

Fig. 4. Ultracentrifuge patterns of glutarinated egg white. (1, 2) Control egg white at 20 and 52 min, respectively; (3, 4) egg white reacted with 15 mols DMGA/mol EWP centrifuged for 64 and 132 min, respectively; (5, 6) egg white reacted with 30 mols DMGA/mol EWP centrifuged for 60 and 128 min, respectively.

Table 5. Performance of glutarinated egg white in angel food cake.

	Meringue specific gravity			Cake volume (ml)		
Mol DMGA/mol EWP	Control	Treatment	Ratio T/C	Control	Treatment	Ratio T/C
3	0.160	0.138	0.86	613	639	1.04
6	0.153	0.133	0.87	596	602	1.01
15	0.148	0.127	0.86	505	285	0.56
30	0.154	0.125	0.81	345 [1]	230 [1]	.....
60	0.154	0.119	0.77	345 [1]	265 [1]	.....
150	0.172	0.118	0.69	240 [1]	230 [1]	.....

[1] Unsatisfactory cake with both control and treated egg white.

reacted with 15 and 30 mols DMGA/mol EWP was 2.83 and 2.95, respectively. This difference probably reflects only very minor, if any, changes in the size and/or shape of the egg white protein molecules. Clearly no major hydrolytic or aggregation changes have occurred under these treatment conditions.

Angel food cake performance

The performance of the glutarinated egg white in angel food cakes is summarized in Table 5. Control cakes were made by diluting unreacted egg white with the same amount of saline as was used in the glutarinated egg white to maintain a constant pH of 9.0 during the reaction. This creates only minor problems at lower levels of reactants, but unsatisfactory cakes resulted from the excessive dilution necessary to maintain the pH at reactant concentrations of greater than 15 mols DMGA/mol EWP.

Since the beating performance is maintained at a high level, the reduction in cake performance is assumed due to alteration of the heat denaturation characteristics of the proteins. This assumption is strengthened by the heat stability data to be discussed.

Emulsifying ability

The emulsifying ability of proteins is an index of their potential use in sausage and related products as a binder and/or stabilizer for the meat emulsion (Pearson *et al.*, 1965). Egg white appears to be capable of emulsifying more fat than the additives normally added to sausages, especially at the pH's typically used in sausage manufacture (Pearson *et al.*, 1965).

Table 6 illustrates that glutarination increases the emulsifying ability of the egg white proteins at pH 4.0, but it has less effect on the emulsifying ability at pH 6.0 and 8.0. Depending upon the pH of the sausage product, glutarination may or may not be beneficial in improving egg white as an emulsion stabilizer in comminuted meat products.

Heat stability

The effect of heating DMGA modified egg white at 60°C for periods up to 15 min on relative viscosity, relative specific gravity of foam, and light transmission are shown in Fig. 5.

The lack of change in the viscosity of unmodified whites differs from those results reported by Seideman *et al.* (1963) and Cunningham *et al.* (1965), who found significant increases upon heating at 60°C. This difference may be due to the protein dilution effect (from *ca.* 19.0 mg N/ml in normal egg white to 16.0 mg N/ml used in these experiments). Ionic strength was maintained at the normal level (*ca.* 0.15*M* NaCl) by diluting with appropriate buffer to negate salt concentration effects on heat stability.

Table 6. Emulsifying ability of glutarinated egg white.

	Gm oil emulsified/mg protein nitrogen		
Mol DMGA/mol EWP	pH 4.0	pH 6.0	pH 8.0
0	13.5	16.2	10.0
3	14.7	14.3	10.6
6	13.9	12.1	10.2
15	16.1	14.1	9.5
30	21.2	12.5	8.3

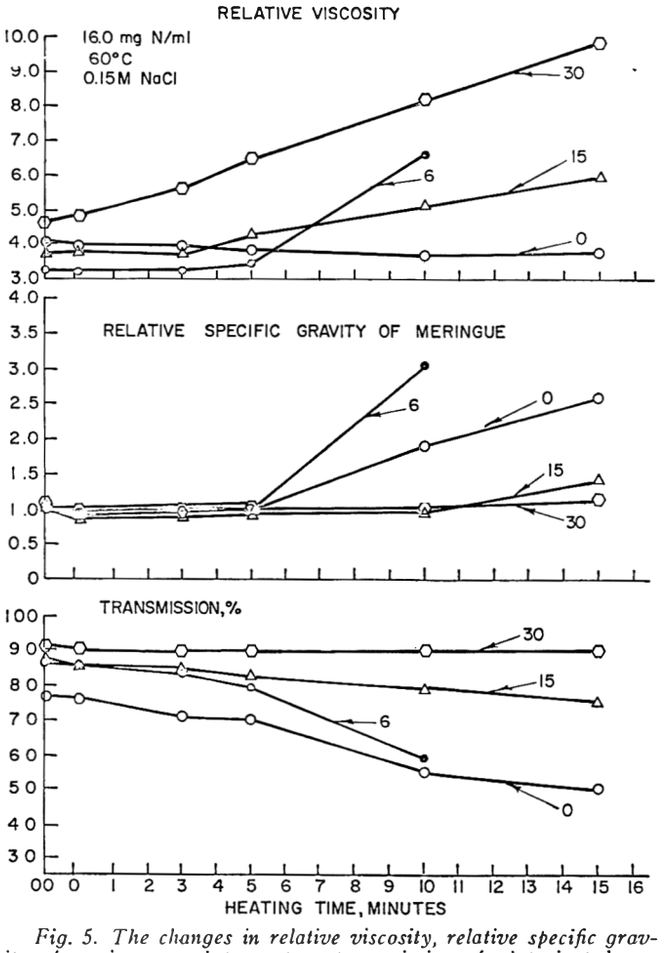

Fig. 5. The changes in relative viscosity, relative specific gravity of meringue, and percentage transmission of glutarinated egg white (0, 6, 15 and 30 mols DMGA/mol EWP) heated for varying time periods at 60°C.

In contrast, foaming power and light transmission properties are markedly affected. The effect of the DMGA at low levels appears to seriously reduce foaming ability, decrease light transmission and sharply increase the viscosity upon heating. A small amount of a fine fibrous precipitate was observed at low levels, which increased with heating. Higher reactant levels yielded a clear solution. The small quantity of the precipitate has not permitted identification. In all instances higher levels offer substantial protection from the heat treatment.

Although no completely satisfactory explanation has been found to explain the inflection point in the quality attribute curves, it does appear that such a point exists near the 3–6 mol DMGA/mol EWP level. Examination of the electrophoretograms in Figs. 1 and 2 confirms this conclusion, as do the emulsifying characteristics given in Table 6 and to a lesser degree the angel cake performance in Table 5.

A possible explanation may be that all the proteins of the egg white carry a negative charge in the pH range 7.5–8.5, where these tests were carried out, due to the addition of the glutaryl groups. This modifies the nature of the protein-protein complexes and subsequently the egg white performance. The significance of the contributions of protein interactions to egg white performance may have

been underestimated. Further chemical modification studies on whole egg white (with little or no dilution) may contribute to our understanding of these functional attributes.

On the basis of the results reported here, 3,3-dimethylglutaric anhydride may offer the egg products technologist a way to control the effect of heat on liquid egg white denaturation, give another tool to permit pasteurization at temperatures more useful for eliminating pathogens from the product, and retain the desired functional properties of the system.

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Dry Layer Permeability and Freeze-Drying Rates in Concentrated Fluid Systems

SUMMARY—Effects of composition, of freezing rates, and of pressure on dry layer permeability of freeze-dried liquids were studied. Permeability was found to be higher for slowly frozen samples of coffee and of a model system, than for rapidly frozen samples. A surface layer of very low permeability was observed in all samples frozen without agitation.

Permeabilities obtained in steady state measurements were found to correlate with permeabilities calculated from freeze-drying rates. A mathematical analysis based on the assumption of a uniform dry layer with constant properties was found to apply better to slush frozen samples than to samples frozen without agitation.

INTRODUCTION

FREEZE-DRYING is usually considerably more expensive than spray drying per unit weight of water removed. High costs are due to investment in equipment, high energy consumption and low capacity because of low rates of drying.

Generally, heat transfer to the sublimation interface and mass transfer from this interface to the condenser or pump are the rate-limiting factors. Proper understanding of these phenomena is essential for equipment and process design.

In this study the water vapor transfer through a dry layer is investigated under conditions of conduction heating where heat transfer is a negligible limiting factor.

One of the first and most comprehensive reviews on freeze-drying foods was made by Harper *et al.* (1957). They found that at a given temperature, the permeability was almost independent of pressure for total pressures lower than 1 mm Hg. They determined the permeability of freeze-dried beef by maintaining ice at a constant temperature on one side, and dry magnesium perchlorate on the other side of a sample plug of known dimensions. They suggested that in liquid foods the permeability was dependent on the freezing rate. In freeze-drying with radiation heating the plot of the square of the fractional weight loss vs. time gave a straight line with intercept on the time axis at 1 hr.

Greaves (1962) found that coffee extracts of high concentration showed apparent eutectics at a lower temperature than dilute solutions.

Mink *et al.* (1962) made a mathematical study of heat and mass transfer in radiation and conduction heating. They presented an empirical equation for the drying time for a process using spiked plates for improved heat transfer.

Lambert *et al.* (1962) made a comprehensive study of heat and mass transfer in the freeze-drying of several products; they also calculated the permeability of several freeze-dried materials. The authors mentioned the existence of a surface film or crust resistance to mass transfer. This resistance decreased with the solids content of the sample. Surprisingly, the permeability decreased during the initial drying period and increased considerably in later stages of freeze-drying of non-fat milk. For this product, the freezing rate had only a slight effect on the permeability, but a sample whose frozen surface was scraped prior to drying had a significantly higher permeability. The solids content of the scrapings was nearly twice that of the bulk. In general, more than half of the moisture was removed at constant rate for several products. The critical moisture content was a function of such factors as the geometry of the sample, the drying rate, and the nature of the sample.

Luyet (1962) recognized the importance of freezing rates on the subsequent freeze-drying process. His studies involved microscopic examination of the frozen and dry materials, types of structure changes during freeze-drying, and rehydration of samples frozen at different rates and temperatures.

Recently, Mackenzie (1966) made an extensive study of the different freezing mechanisms and their relations to the freeze-drying process. He found that for each system there is a characteristic temperature above which freeze-drying is not possible without collapse of the structure. This temperature was usually much lower than the eutectic temperature, and was very similar to the critical recrystallization temperature, which could be increased by adding substances with high transition temperatures, such as dextran.

Rey (1963) observed that fast freezing resulted in the formation of small crystals and products of high quality, but that very rapid freezing resulted in a splitting of the sample due to stresses caused by steep temperature gradients.

Mackenzie *et al.* (1963) found that very rapidly frozen 30% gelatin gel freeze-dried faster at −30°C when permitted to recrystallize, confirming observations by other authors that large crystals result in increased drying rates. These authors also showed that more shrinkage occurred in the non-recrystallized samples during freeze-drying.

Burke *et al.* (1964) mentioned that the freezing operation might be as important as the drying process. These authors also observed a constant and a falling rate period during freeze-drying of several products, confirming earlier observations made by Lambert *et al.* (1962).

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Hardin (1965) made an exhaustive mathematical analysis of the freeze-drying process. He found that, theoretically, the drying times at 0.5 or at 2 mm Hg chamber pressure should not differ significantly. In practice, however, the drying time at 2 mm Hg was considerably longer. Hardin also made the interesting experiment of measuring the pressure profile within the dry layer to prove theoretical predictions.

Dyer (1965), too, made a study of the mathematical relations governing the freeze-drying process. He observed that at −20°C, beef had a water vapor pressure of 0.5 mm Hg, while the vapor pressure of pure ice was 0.7 mm Hg. He observed only small differences in the drying rates at chamber pressures below 0.8 mm Hg, thus confirming previous observations made by Harper *et al.* (1957).

Nei *et al.* (1964) observed that dehydration proceeded faster in the slowly frozen material during the primary phase of drying, but more rapidly in the quickly frozen sample during the final stage.

EXPERIMENTAL PROCEDURE

Direct determination of the permeability

For direct determination of permeability the suspensions were deaerated and then frozen in glass cylinders of 25 mm ID and 20 mm height glued to flat aluminum foil. The freezing rates employed were:

Very slow freezing: free convection freezing at −5°C.

Slow freezing: free convection freezing at −20°C.

Fast freezing: freezing at −40°C with air agitation.

Very fast freezing: freezing with liquid N_2 from bottom of the glass cylinder.

Slush freezing: freezing at −20°C with intermittent manual agitation; the slush was transferred to molds and then solidified at −40°C.

Freezing the liquid sample by immersion into liquid N_2 yielded a cracked sample with no mechanical strength.

After freezing, the samples were dried at a surface temperature of −25°C until the free water had been removed. Then the temperature was raised to +20°C to remove most of the adsorbed water. The dry cylinders suffered a contraction of about 1 mm making it easy to remove them from the mold. The area of the samples was 4.90 cm^2 and the height varied between 10 and 18 mm.

Samples of coffee (20% and 30% solids) as well as of the model system (10% glucose, 10% microcrystalline cellulose; 2% potato starch) showed enough mechanical strength to be handled without breaking.

For the permeability determination, the sample cylinder was wrapped in a piece of plastic insulating tape to cover all the lateral surface without leaving space between the tape and the sample. This new cylinder was then attached to the sample holder, as shown in Fig. 1, and introduced into the experimental freeze-drier (Fig. 2).

In this set-up, ice at constant temperature (corresponding to vapor pressures of 0.70 to 0.80 mm Hg) could be maintained on one side of the sample, while on the other side the water could be condensed in a weighed flask cooled with liquid N_2 and connected to a vacuum pump.

Steady state had to be attained in relation to the water adsorbed on the surface of the sample. During the first 10 min a temperature rise of 10°F occurred in the center of the sample due to water vapor adsorption, and after 100 min a constant moisture content profile was established within the sample, after which time the water vapor transfer occurred at constant rate and temperature. The rate was determined by weighing the amount of water condensed during a period of 3 to 15 hr.

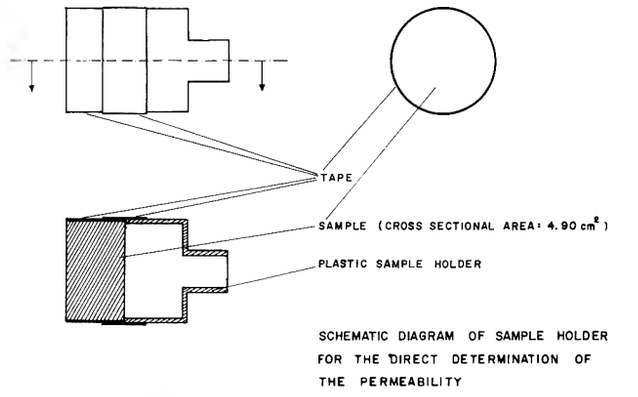


Fig. 1. Schematic diagram of sample holder for direct determination of permeability.

The permeability was then calculated using the known values of the area, thickness of the sample, and water vapor pressure differences between the two sides of the sample. A term for the resistance to mass flow of the system, which was determined with no sample, was included in the calculations which are shown in Fig. 3.

After one determination the sample holder with the sample could be removed, the upper surface layer (0.5 to 1 mm) scraped off, and a new determination performed.

Properties of all samples, such as moisture content profile, cracking, texture, possible melting, were examined after completing the last determination.

Rates of freeze-drying

The same experimental set-up shown in Fig. 2 was used. In this case the sample was frozen in a circular dish of 45.3 cm^2 area and 1.2 cm height with Lucite (trade mark of E. I. DuPont de Nemours Co.) side wall and flat brass bottom.

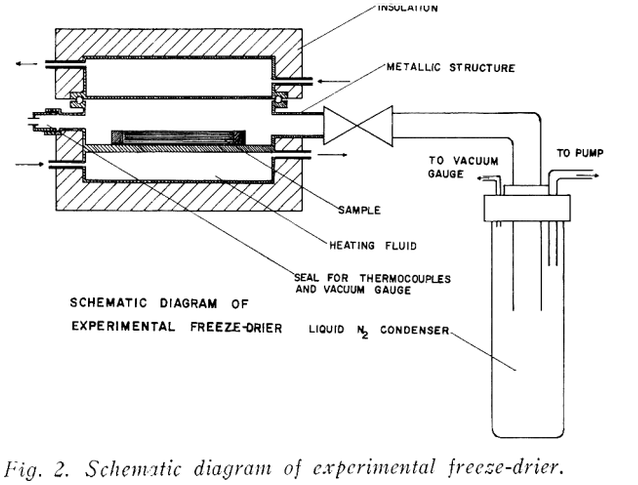
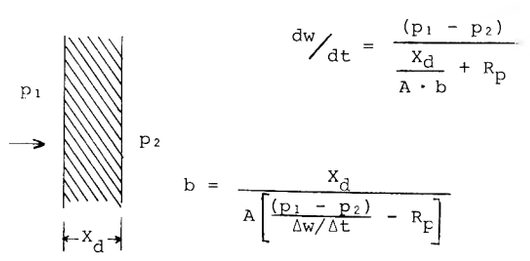


Fig. 2. Schematic diagram of experimental freeze-drier.

PERMEABILITY



where:

b - permeability, g/sec cm mm Hg

X_c - thickness, cm

A - area, cm^2

$(p_1 - p_2)$ - water vapor pressure differential, mm Hg

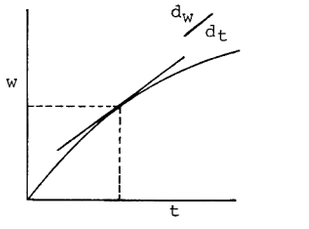
$\frac{dw}{dt}$ - flow rate of water vapor, g/sec

R_p - resistance of pipe to mass flow, sec mm Hg/g

$R_p = 1.7 \times 10^2$ sec mm Hg/g

Fig. 3. Calculations for the direct determination of permeability.

DETERMINATION OF PERMEABILITY DURING FREEZE-DRYING



$$X_d = \frac{w}{\rho \cdot X_{H_2O} \cdot A}$$

$$\text{Slope} = \frac{dw}{dt}$$

$$\therefore \quad b = \frac{X_d}{A\left[\frac{(p_1 - p_2)}{dw/dt} - R_p\right]}$$

where:

X_d - thickness of dry layer, cm

w - weight of water condensed, g

ρ - density of sample, g/cm^3

X_{H_2O} - weight fraction of water in sample

A - sublimation area of sample, cm^2

$(p_1 - p_2)$ - water vapor pressure differential, mm Hg

$\frac{dw}{dt}$ - drying rate, g/sec

b - permeability, g/sec cm mm Hg

P_p - resistance of pipe to mass transfer, sec mm Hg/g

Fig. 4. Calculations for the indirect determination of permeability.

The thickness of the samples varied from 0.9 to 1.2 cm and thermocouples were placed at different points. Heat was transferred by conduction through the brass bottom and the frozen layer to the sublimation interface.

The weight of water removed at any time could be determined. By graphical differentiation the drying rate at any time was calculated. The thickness of the dry layer was determined from the weight of water removed. Details of the calculation of the permeability are shown in Fig. 4.

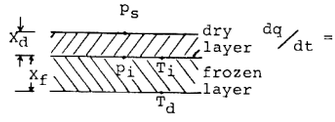
For the freeze-drying rate determinations only 20% coffee (Maxwell House, trade mark of General Foods Corp.) solutions and a model containing 10% glucose, 10% microcrystalline cellulose (Avicel, trade mark of FMC Corp.), and 2% potato starch (boiled) were used. The latter proved to be an excellent model for the study of mass transfer phenomena during freeze-drying.

The driving force for mass transfer through the dry layer was the difference between the vapor pressure at the sublimation interface temperature and the vapor pressure at condenser temperature (−196°C). Although the interface temperature (T_i) was considered equal to the bottom temperature (T_d), the calculations in Fig. 5 show that this introduces a negligible error for the dimensions involved.

RESULTS AND DISCUSSION

Direct permeability determination

Some significant results obtained in permeability studies are shown in Figs. 6 and 7. Extensive study of the surface layer resistance showed that it could amount to the



$$\frac{dq}{dt} = \frac{A \cdot b \cdot (p_i - p_s)}{X_d} \Delta H_s \qquad (1)$$

$$\frac{dq}{dt} = \frac{A \cdot K_f \cdot (T_d - T_i)}{X_f} \qquad (2)$$

Combining (1) and (2)

$$T_d = \frac{b \cdot X_f}{K_f \cdot X_d}(p_i - p_s)\,\Delta H_s + T_i \qquad (3)$$

if:

T_i - interface temperature = -25°C

p_i - interface water vapor pressure = 0.48mm Hg

p_s - surface water vapor pressure = 0.00mm Hg

ΔH_s - latent heat of sublimation = 650 cal/g

b - permeability of dry layer = 2×10^{-5} g/sec·cm·mm Hg

K_f - thermal conductivity of frozen layer = $0.005 \frac{\text{cal}}{\text{sec cm}}\,^{\circ}\text{C}$

X_f - thickness of frozen layer = 1 cm

X_d - thickness of dry layer = 0.5 cm

Then:

$T_d = -23^{\circ}\text{C}$

Note: X_d = 0.5 cm is the thickness of the surface layer in terms of equivalent resistance.

Fig. 5. Temperature at interface.

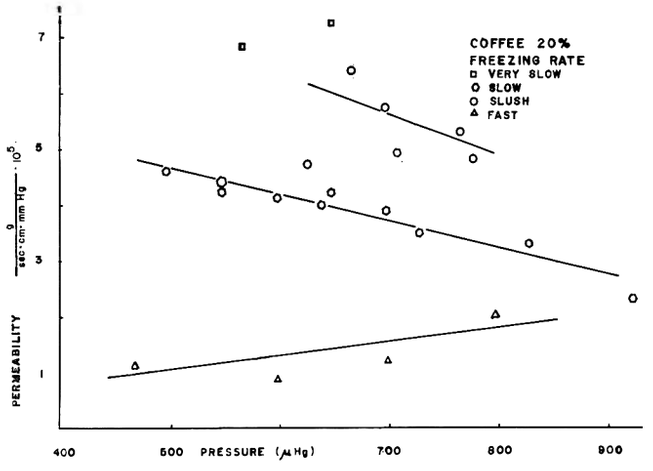


Fig. 6. Permeability of freeze-dried coffee as a function of freezing rate and chamber pressure.

resistance to mass transfer equivalent to 0.3 to 1.4 cm of the normal dry layer. The value of this resistance could not be correlated properly with the freezing process, except in the case of slush freezing where the surface resistance was very small. Cracks in the surface and the relative humidity of the freezing room might have influenced the surface resistance.

After the permeability determination, a moisture content gradient could be determined by observing the plasticity of the sample on the high pressure side and its brittleness on the low pressure side.

The permeability was not affected by the relatively high moisture content at the high pressure side. After steady state had been attained the sample cylinder of 20% coffee showed an average moisture content of 0.05 g H_2O/g solids.

The thickness of the sample cylinder could not be determined better than within ± 0.5 mm. This, then, is the major source of error in the permeability determination. An analysis of the error propagation—including other variables such as weight, flow area, and driving force determination—showed that the maximum probable error is $\pm 0.5 \times 10^{-5} \frac{g}{\text{sec cm mm Hg}}$.

Figs. 6 and 7 show the weak total pressure dependence of the permeability below 0.80 mm Hg, a finding in agreement with results obtained by Harper *et al.* (1957) and by Dyer (1965).

The results obtained in this study show clearly that the permeability of the dry layer is markedly affected by the freezing of the liquid. Slush freezing was actually a method of slow freezing, but it resulted in a much higher permeability. This can be explained by the virtual absence of surface layer resistance and by incorporation of air into the slush. Fast freezing gave the lowest permeability due to the formation of very small crystals of water. Very slow freezing resulted in the formation of long ice crystals in bundles and in different directions as observed in the freeze-dried sample.

The importance of freezing rates on subsequent drying was qualitatively recognized and discussed by Luyet (1962) and many other authors. Although Lambert *et al.* (1962) found only a slight effect on the permeability of non-fat milk, these authors did not specify the freezing rates used in their work.

Freeze-drying rates

The freeze-drying process was analyzed in terms of plots of the apparent permeability vs. the thickness of the dry layer. Figs. 8 and 9 show that for 20% coffee and the model system, slush freezing gave significantly higher permeabilities than did slow freezing. The higher initial permeablity of the slush frozen samples due to the virtual absence of the very impermeable surface layer is important.

Removing this layer from the frozen sample increased the initial permeability and the drying rate during the whole period. This confirms a prevous observation by Lambert *et al.* (1962). These authors, however, found a high initial permeability which first decreased with the thickness of the dry layer, then subsequently increased. This finding is contrary to the results presented in this paper.

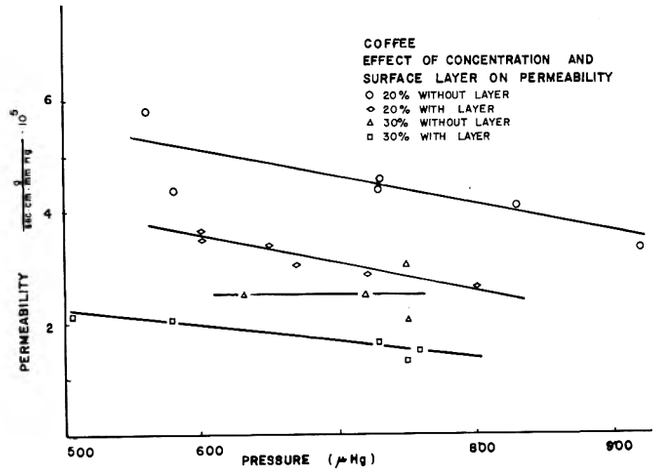


Fig. 7. Effects of concentration and of the presence of a surface layer on the permeability of freeze-dried coffee.

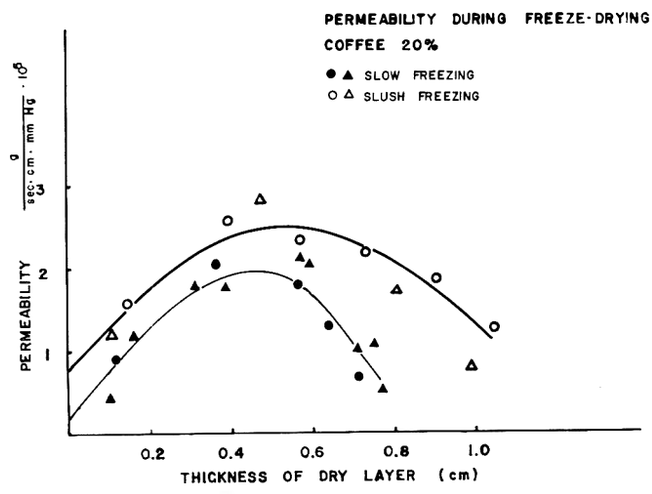


Fig. 8. Permeability of freeze-dried coffee estimated from freeze-drying rates.

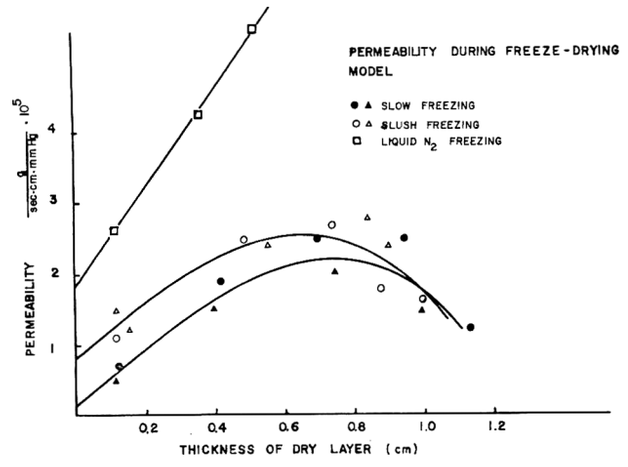


Fig. 9. Permeability of a freeze-dried model system estimated from freeze-drying rates.

The shape of the curves found may be explained by the high proportion of the surface resistance to the total resistance during the initial period of drying, resulting in a low apparent permeablity. During later periods the dry layer thickness increases and accounts for most of the mass flow resistance, resulting in a higher apparent permeability. Finally, the apparent permeability again decreases due to factors such as impaired heat transfer, irregular heating, edge effects. Therefore, the plot only represents reality well up to a dry layer thickness of 0.7 cm.

Note the irregularity of the permeability vs. dry layer thickness plot for the model system frozen in liquid N_2. Extensive cracking of the sample occurs and the water vapor can escape. In this case, the permeability can hardly be used for any mathematical analysis.

Fig. 10 shows schematically how the frozen layer recedes for each of the freezing processes. Very fast freezing gives a completely irregular interface. The fairly irregular surface for the slow frozen sample is understandable because the rate of heat removal from the sample during freezing is inversely proportional to the thickness of the frozen layer. If the convection heat transfer coefficient is large, the sample freezes slower at the center. This results in higher permeability at the center. The geometry and composition of the sample are also responsible for a heterogeneity in crystal size and orientation.

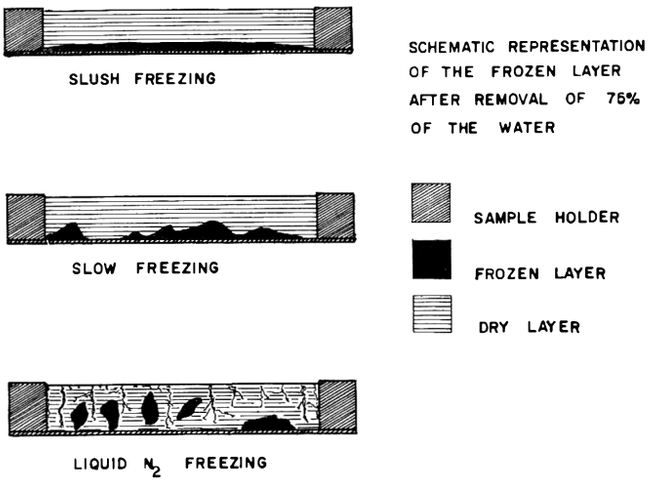


Fig. 10. Schematic representation of the frozen layer after removal of 75% of the water.

The slush frozen samples showed a uniform texture due to random distribution of the ice crystals. The frozen interface receded very uniformly.

During the selection of an appropriate model system, several compositions were studied and it was observed that 20% sucrose solutions could not be dried at −20°C, which is well below the eutectic temperature. Similar observations were made by Mackenzie (1966). In the present study, adding microcrystalline cellulose and heated potato starch stabilized the structure and made possible the freeze-drying of a system containing high sugar concentrations.

Twenty % coffee solutions could be freeze-dried at −20°C, but 40% solutions showed melting spots at this temperature, although the nature of the solutes was the same at both concentrations. It appears that the concentrated solutions have a lower apparent eutectic point, a finding made earlier by Greaves (1962).

During freezing, a viscous layer is formed on the surface of the sample of coffee. At −16°C this layer can be scraped off; then drying proceeds faster and can be performed at higher temperatures.

A sample from the surface had a moisture content of only 1.9 g H_2O/g solids while the moisture content of the bulk was 4.0 g H_2O/g solids. No difference was observed in the composition of these fractions as determined by the refractive index of equal concentration solutions.

In freeze-drying of samples which developed a surface layer, a constant rate period was observed. Other authors, including Lambert *et al.* (1962) and Burke *et al.* (1964) have also observed constant rate periods, and have analyzed freeze-drying data on the basis of existence of a constant rate and a falling rate period. Our experiments show that a constant drying rate may be due to the surface resistance which is the major component of the resistance to mass flow during the initial period of drying, at least under conditions in which heat transfer is not limiting. This means that the critical moisture content is dependent on the freezing process, geometry of the sample, and method of preparation.

Dyer (1965) showed that a surface layer of low permeability is formed at the surface of meat. In our experiments a shorter constant rate period was observed for slush frozen samples. Harper *et al.* (1957) mentioned that for beef, the plot of the square of the fractional weight loss vs. time gave an intercept of the time axis at 1 hr. This finding, too, can be attributed to possible surface resistance.

Several insoluble and partially soluble organic solvents were added to coffee solutions before freezing to form a frozen solvent layer of the top surface. Benzene reduced the surface resistance to water vapor flow, although not as effectively as did mechanical removal of the surface layer.

Table 1. Permeability of freeze-dried systems.

Treatment	Direct determination	Permeability $\frac{g}{\text{sec-cm mm Hg}} \times 10^5$ Maximum during freeze-drying
Coffee, slow freezing	4.0	2.0
Coffee, slush freezing	6.5	2.8
Model, slow freezing	....	2.0
Model, slush freezing	3.4	2.6

Model composition: 10% microcrystalline cellulose, 2% potato starch.

Drying from the sides and from the bottom was a frequent source of irregularities in the drying curves. This problem was much less serious for the model system than for coffee. Dyer (1965), who experienced a similar problem, used several binding materials to get good thermal contact between the heat source and the sample. It can be expected that in commercial practice good thermal contact will be difficult to obtain.

In Table 1 representative values of the permeability constant obtained by the direct method and from the drying process are shown. Note that in the direct method the determination was made at −20°C, while for the freeze-drying process the temperature was −25°C. This temperature difference could be responsible for the differences in permeability values. The water vapor pressure at the interface was obtained from the temperature for pure ice. Dyer (1965) has shown that the solutes present lower the vapor pressure of the frozen mixture.

CONCLUSIONS

1. Slow freezing and slush freezing of fluid systems gave permeabilities several times higher than those obtained with fast freezing.

2. During freezing of most liquid systems a very impermeable surface layer of resistance to mass flow equivalent to 0.3 to 1.4 cm of the normal dry layer is formed.

3. Slush freezing or mechanical removal of the surface layer at a suitable temperature reduces the surface resistance.

4. Only in the case of slush frozen samples can the drying time be predicted by mathematical models. In the case of anistropic distributions of ice crystals, such as exist in samples frozen by other methods, the predictions are inadequate.

5. In general, permeability increases with increasing size of ice crystals.

6. A model containing 10% glucose, 10% microcrystalline cellulose, and 2% potato starch was found useful for the study of mass and heat transfer at low temperatures.

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Fatty Acid Composition of the Inner and Outer Layers of Porcine Backfat as Affected by Energy Level, Sex and Sire

SUMMARY—The effects of energy level, sex and sire upon the fatty acid composition of porcine fackfat were studied in two separate experiments. In both experiments, the inner layer of backfat consistently contained more total saturated fatty acids, primarily accounted for by stearic acid, and contained less palmitoleic, oleic, and linoleic acids than the outer layer. None of the variables in either experiment significantly ($P > .05$) affected the differences between layers.

In Experiment 1, energy levels below 80% of full feed significantly ($P < .01$) decreased stearic acid content and increased linoleic acid content. In Experiment 2 the fatty acid composition of backfat from pigs fed 80% of full feed did not differ significantly ($P > .05$) from full-fed pigs.

Backfat from spayed gilts had significantly ($P < .01$) less linoleic acid than that from boars or barrows, while boars had a significantly ($P < .01$) higher content of linoleic acid than the other sexes. In Experiment 1, the backfat from barrows contained significantly ($P < .01$) more stearic acid than that from gilts.

The data from both experiments indicated that sire significantly ($P < .01$) affected the linoleic acid content of the backfat, while the results of Experiment 2 also showed significant ($P < .01$) sire effects upon the palmitic, palmitoleic, and stearic acid contents.

INTRODUCTION

LIMITED FEEDING STUDIES with hogs have generally resulted in decreased fat deposition with a concomitant increase in lean yield. Hilditch *et al.* (1939), Shorland *et al.* (1945) and Merkel *et al.* (1958) reported that fat deposition of restricted-fed hogs was not only slower, but the fat was softer than full-fed hogs. Hilditch *et al.* (1939) and Shorland *et al.* (1945) indicated that this was possibly due to decreased palmitic acid and increased deposition of linoleic acid. This was substantiated by Dahl *et al.* (1965) who reported evidence for preferential deposition of polyunsaturated fatty acids, especially linoleic acid.

Greer *et al.* (1965) observed that the degree of saturation decreased and linoleic acid increased in the outer layer of backfat of pigs restricted to 85% of full feed. They noted that further feed restriction to 70% caused a decrease of linoleic acid from that obtained at 85% of full feed. These same authors reported that palmitic acid decreased as the feed level was restricted to 85%, but that it increased as feed level was further restricted to 70%.

Lush *et al.* (1936) indicated that the backfat of gilts had higher iodine values (1.7 units) than that of littermate barrows. Johns (1941) reported that iodine values of backfat ranged in decreasing order from boars to gilts, barrows and spayed gilts. He reported that the iodine values varied inversely with the quantity of backfat among all sexes. This same author observed that the backfat from boars contained a higher level of linoleic acid than gilts, barrows or spayed gilts. Koch *et al.* (1968) found that backfat from barrows contained more palmitic and stearic acids and less linoleic acid than that of gilts.

Few data are available concerning heritability of the fatty acid composition of porcine backfat. However, Lush *et al.* (1936) indicated that the variance in fat firmness between litters sired by the same boar was slightly larger than the variance between the progeny of different boars within the same year.

There has been some disagreement as to which layer of backfat is more readily altered in its fatty acid composition. Sink *et al.* (1964) reported that the saturated acids (especially palmitic and stearic acids) are preferentially deposited in the inner layer of backfat. These workers further indicated that the levels of palmitoleic, oleic and linoleic acids decreased as degree of saturation increased. Garton *et al.* (1952) indicated that diet had a greater effect upon the fatty acid composition of the outer layer of backfat than upon the inner layer.

However, Bhattacharya *et al.* (1931) reported that diet had more effect on the inner layer. Hilditch *et al.* (1940) reported that in the early stages of inanition, a preferential selection occurs from the reserves of the outer backfat layer, but that prolonged starvation caused the largest degree of mobilization from the inner layers.

In view of these observations, the present investigation was undertaken to study the fatty acid composition by gas-liquid chromatography in the inner and outer layers of porcine backfat as a result of: 1) energy level; 2) sex; and 3) sire.

EXPERIMENTAL

TWELVE BARROWS AND NINE GILTS of Yorkshire breeding from three different sires were used in Experiment 1. These pigs (one from each sire) were allotted at approximately 100 lb live weight to each of the following seven treatments: gilts and barrows fed 3 lb of basal ration (B, Table 1); gilts and barrows fed 3 lb of basal ration plus 1 lb of corn starch (B + 1); gilts and barrows fed 3 lb of basal ration plus 2 lb of corn starch (B + 2); and barrows fed 3 lb of basal ration plus 3 lb of corn starch (B + 3).

When the pigs in each lot averaged 125, 150, and 175 lb live weight, respectively, the starch portion of the ration was increased by 0.5 lb increments, respectively. The highest levels of daily feed intake for each full-fed sex group were similar to previous observations for full-fed

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Table 1. Composition of the rations.

Ingredients	Experiment 1	Experiment 2	
	Basal ration[1]	Basal ration (weaning to 125 lb)	Basal ration (125 lb to slaughter wt)
	%	%	%
Corn	52.8	77.7	86.8
Soybean meal (50% crude protein)	40.0	15.0	6.5
Meat and bone scraps (50% crude protein)	5.0	2.5	2.5
Alfalfa meal (17% crude protein)	.....	2.5	2.5
Limestone	0.3	0.6	0.5
Dicalcium phosphate	0.4	0.2	0.2
Trace mineral salt (hi-Zn)	0.5	0.5	0.5
VATM (vitamin premix)	1.0	0.5	0.5
Calculated analysis:			
Protein	27.0	16.0	12.4
Fat[2]	2.6 to 3.0	3.9 to 4.0	4.1 to 4.3
Ca	0.9	0.7	0.6
P	0.8	0.5	0.4

[1] Energy of basal ration as determined by bomb calorimetry was 1.905 therms/lb; energy of the corn starch was 1.716 therms/lb.
[2] % fat was calculated from Morrison (1957).

pigs. Gross energy of the basal ration and the corn starch was determined by bomb calorimetry.

In Experiment 2, four Yorkshire X Hampshire crossbred litters each containing four boars and four gilts were assigned, two weeks post-weaning, to four sex groups: boars, barrows, gilts and spayed gilts. The necessary castration was performed at this time. Each litter was represented in each sex group by two pigs. One pig from each litter and sex group was fed *ad libitum* (100%) and the other pig was restricted to 80% of full feed (see Table 1 for the composition of the rations). The daily feed intake of the restricted lots was adjusted weekly from the feed consumption of the respective *ad libitum* lots.

All pigs from both experiments were slaughtered when they reached market weight (approximately 225 lb). The carcasses were allowed to chill at 3°C for 24 hr. Backfat samples were excised from the tenth rib region, placed in polyethylene bags, sealed, frozen and stored at −30°C until analyzed for fatty acid composition. At the time of analysis the backfat samples were divided into inner and outer layers at the visible connective tissue septum. Methyl esters of fatty acids were prepared according to a rapid, low temperature method introduced by McGinnis *et al.* (1965).

A Barber-Colman, Model 20, gas chromatograph, equipped with a radium ionization detector, was used with the following settings: argon gas flow rate, 154 ml/min; injector port and detector temp, 240°C; column temp, 175°C; cell voltage, 1250 V; and sensitivity, 1×10^{-7} amp full scale. The column, 6 ft × ¼ in. (outside diameter) copper tubing, was packed with 12% ethylene glycol succinate on 60/70 mesh Anakron A.

The fatty acids were identified by comparison with methyl ester standards and the proportion of fatty acids present was calculated by integrating the area under the peaks.

Small amounts (< 1.0%) of fatty acids other than myristic, palmitic, palmitoleic, stearic, oleic and linoleic acids were observed, but are not included in these results.

The statistical procedures used were those outlined by Steel *et al.* (1960).

RESULTS AND DISCUSSION

In experiment 1, energy levels below 80% of full feed resulted in marked changes in the fatty acid composition of backfat (Table 2). The lowest energy levels (57% and 67% for barrows and gilts, respectively) produced significantly ($P < .01$) less saturated fat than that from the corresponding full-fed pigs. Stearic acid accounted for most of this decrease in total saturated fatty acids.

Among gilts, palmitic acid content also significantly ($P < .05$) decreased as energy level decreased. However, among barrows the palmitic acid content of the backfat increased slightly in the 86% and 72% of full feed lots compared to the full-fed barrows, but then decreased significantly ($P < .05$) as feed was restricted to 57% of full feed. There were no significant ($P > .05$) differences in myristic acid content for either barrows or gilts attributable to energy level, although this fatty acid tended to increase at first and then decrease as energy level decreased.

Linoleic acid increased significantly ($P < .01$) as energy decreased. This effect became apparent with less energy restriction among gilts than among barrows. Additionally, restricted energy levels among gilts produced a significantly ($P < .05$) higher content of palmitoleic acid. There were no significant ($P > .05$) differences in oleic acid content of porcine backfat attributable to energy level for either barrows or gilts. The results of Experiment 2 (Table 2) indicate that there were essentially no differences ($P > .05$) in the fatty acid composition of backfat between full-fed pigs or those limited to 80% of full feed.

The effects of energy level upon the fatty acid composition of porcine backfat can best be explained by the work of Dahl *et al.* (1965) who reported that the polyunsaturated fatty acids are preferentially deposited. Additionally, as the level of feed or energy is restricted, less fat will be deposited, and that which is deposited is more unsaturated with especially more linoleic acid being laid down (Hilditch *et al.*, 1939 and Greer *et al.*, 1965).

The linoleic acid content of backfat from the spayed gilts was significantly ($P < .01$) lower than that from boars or barrows (Experiment 2, Table 2). The backfat of spayed gilts displayed a significantly ($P < .05$) higher content of total saturated fatty acids than that of the other sexes. The backfat of boars contained a significantly ($P < .01$) greater amount of linoleic acid than that from barrows, gilts or spayed gilts.

In Experiment 1, the fatty acid composition of the backfat from only the full-fed barrows and gilts can be compared. Only stearic acid, was significantly ($P < .01$) different between barrows and gilts.

These sex effects may be attributed to the obvious hormonal differences or possibly to the actual differences in amount of backfat deposited. With regard to the latter supposition, the full-fed barrows in Experiment 1 had an average backfat thickness of 1.62 in., while the gilts averaged 1.38 in. In experiment 2 the average backfat thick-

Table 2. The effect of sex and energy level upon the fatty acid composition of porcine backfat.[1]

	Experiment 1						
	Barrows				Gilts		
Fatty acid:[2]	B+3 (100%)[3,4]	B+2 (86%)	B+1 (72%)	B (57%)	B+2 (100%)[5]	B+1 (83%)	B (67%)
14:0, %	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
16:0, %	23.0 ± 0.8^{a,b}	23.8 ± 0.8^{b}	24.1 ± 0.8^{b}	21.3 ± 0.7^{a}	23.2 ± 0.8^{a,b}	22.3 ± 0.8^{a,b}	21.4 ± 0.7^{a}
16:1, %	3.5 ± 0.2^{a,b}	4.0 ± 0.3^{a,b}	3.5 ± 0.3^{a,b}	3.6 ± 0.2^{a,b}	3.4 ± 0.2^{a}	4.0 ± 0.3^{a,b}	4.2 ± 0.2^{b}
18:0, %	18.6 ± 0.5^{c}	15.4 ± 0.4^{a,b}	15.4 ± 0.4^{a,b}	14.9 ± 0.5^{a,b}	16.7 ± 0.6^{b}	15.5 ± 0.5^{a,b}	13.7 ±0.4^{a}
18:1, %	46.4 ± 0.9	47.1 ± 0.8	46.6 ± 0.8	47.2 ± 0.8	47.8 ± 0.8	45.9 ± 0.7	47.2 ± 0.8
18:2, %	7.3 ± 0.5^{a}	8.5 ± 0.5^{a}	8.8 ± 0.6^{a}	11.8 ± 0.7^{b}	7.9 ± 0.5^{a}	11.2 ± 0.6^{b}	12.3 ± 0.6^{b}
Total saturated, %	42.8 ± 0.9^{d}	40.4 ± 0.9^{b,c,d}	40.8 ± 0.9^{b,c,d}	37.4 ± 0.8^{a,b}	41.0 ± 0.8^{c,d}	38.9 ± 0.8^{a,b,c}	36.3 ± 0.7^{a}

	Experiment 2					
	Level of full feed		Sex			
Fatty acid:	100%	80%	Boars	Barrows	Gilts	Spayed gilts
14:0, %	0.9 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.2
16:0, %	19.6 ± 0.3	19.4 ± 0.3	19.2 ± 0.6	19.5 ± 0.7	19.2 ± 0.7	20.0 ± 0.8
16:1, %	3.7 ± 0.5	3.5 ± 0.5	3.7 ± 0.5	3.6 ± 0.5	3.8 ± 0.4	3.5 ± 0.6
18:0, %	14.9 ± 0.7	14.9 ± 0.7	15.0 ± 0.8	14.6 ± 0.7	14.8 ± 0.7	15.1 ± 0.8
18:1, %	46.2 ± 0.8	46.5 ± 0.8	45.6 ± 0.8	46.6 ± 0.9	46.0 ± 0.9	47.1 ± 0.9
18:2, %	14.8 ± 0.7	14.9 ± 0.6	15.6 ± 0.7^{c}	14.9 ± 0.7^{a,b}	15.3 ± 0.6^{b}	13.4 ± 0.6^{a}
Total saturated, %	35.3 ± 0.8	35.1 ± 0.9	35.0 ± 0.8^{a}	34.9 ± 0.9^{a}	35.0 ± 0.8^{a}	35.9 ± 0.9^{b}

[1] All means with the same superscripts or those without superscripts are not significantly different ($P > .05$).
[2] Carbon chain length:number of double bonds. Fatty acids are expressed as % of total methyl esters.
[3] Mean ± standard error of the mean.
[4] The full-fed barrows (100%) received the basal ration (3 lb) plus 3 lb of corn starch daily; the 86% of full feed barrows received the basal (3 lb) plus 2 lb of corn starch daily, etc.
[5] The full-fed gilts (100%) received the basal ration (3 lb) plus 2 lb of corn starch daily, etc.

ness for boars, barrows, gilts and spayed gilts was 1.04, 1.31, 1.16 and 1.28 in., respectively.

From the results of White *et al.* (1964) and Dahl *et al.* (1965), it appears that as the amount of backfat increases, a smaller proportion of the fat is derived from the preferentially deposited polyunsaturated fatty acids and a greater proportion of saturated fatty acids is derived from the nonlipid diet components. This relationship between amount of backfat and fatty acid composition in the present study generally supports the above postulation.

A significant ($P < .05$ and $P < .01$ in experiments 1 and 2, respectively) difference in linoleic acid content attributable to sire was observed (Table 3). In addition, in Experiment 2, palmitic, palmitoleic, and stearic acid contents of the backfat were significantly ($P < .01$) affected by sire. The pigs of sire 4 exhibited a significantly ($P < .01$) higher content of linoleic and palmitoleic acids than those from the other sires. However, the pigs of sire 2 exhibited a higher linoleic acid content than those from sires 1 and 3, but the palmitoleic acid content was lower than that from sires 1 and 3.

The pigs of sire 4 also exhibited a significantly ($P < .05$) lower content of stearic acid. Those of sire 2 exhibited the highest content of stearic acid and displayed the lowest content of palmitic acid. Despite statistically significant differences in the individual fatty acid percentages, the degree of saturation remained fairly constant between sires. This is evidenced by the nonsignificant ($P > .05$) difference in total saturated fatty acids between sires.

Although there were no significant ($P > .05$) sire differences for total saturated fatty acids in Experiment 1, degree of saturation was inversely related to linoleic acid content. A similar, but less marked, trend was also observed in Experiment 2. Since energy level was balanced within sires, and since Dahl *et al.* (1965) reported a selective deposition of polyunsaturated fatty acids, these data indicate that the ability to selectively deposit unsaturated fats, particularly linoleic acid, might be heritable.

The data in both experiments indicate that the inner layer of backfat was more saturated than the outer layer (Table 4). The inner layer of backfat from the pigs in both experiments contained significantly ($P < .01$) more stearic acid and less palmitoleic, oleic, and linoleic acids than the outer layer. There were no significant ($P > .05$) differences for myristic or palmitic acid between backfat layers in either experiment. These data agree with the results reported by Sink *et al.* (1964).

The analysis of variance of these data indicated that no interaction ($P > .05$) was observed between the variables (energy level, sex and sire) and the fatty acid composition of the two backfat layers. These data indicate that the fatty acid composition of porcine backfat layers are distinctly different and these inherent differences between layers are greater than the effects of energy level, sex or sire.

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Table 3. The effect of sire upon the fatty acid composition ofporcine backfat.[1]

	Experiment 1		
	Sire		
Fatty acid:[2]	1[3]	2	3
14:0, %	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
16:0, %	23.3 ± 0.2	22.5 ± 0.3	22.4 ± 0.3
16:1, %	3.5 ± 0.1	3.8 ± 0.1	4.0 ± 0.1
18:0, %	16.0 ± 0.5	15.9 ± 0.5	15.4 ± 0.5
18:1, %	47.0 ± 0.7	47.0 ± 0.7	46.6 ± 0.8
18:2, %	9.0 ± 0.3[a]	9.7 ± 0.4[a,b]	10.4 ± 0.4[a,b]
Total saturated, %	40.5 ± 0.8	39.6 ± 0.8	38.9 ± 0.9

	Experiment 2			
	Sire			
Fatty acid:	1	2	3	4
14:0, %	0.8 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.9 ± 0.2
16:0, %	19.5 ± 0.7[b]	18.9 ± 0.6[a]	19.7 ± 0.9[b]	19.8 ± 0.7[b]
16:1, %	3.6 ± 0.5[a]	3.3 ± 0.4[a]	3.6 ± 0.4[a]	4.0 ± 0.4[b]
18:0, %	14.8 ± 0.5[b]	15.9 ± 0.5[c]	14.9 ± 0.5[b]	14.0 ± 0.4[a]
18:1, %	47.0 ± 0.7	46.2 ± 0.6	46.7 ± 0.6	45.4 ± 0.6
18:2, %	14.3 ± 0.3[a]	14.9 ± 0.3[a]	14.2 ± 0.3[a]	15.9 ± 0.3[b]
Total saturated, %	35.2 ± 0.9	35.6 ± 0.8	35.4 ± 0.7	34.7 ± 0.7

[1] All means with the same superscripts or those without superscripts are not significantly different ($P > .05$).
[2] Carbon chain length:number of double bonds. Fatty acids are expressed as % of total methyl esters.
[3] Mean ± standard error of the mean.

Table 4. The effect of backfat layer upon the fatty acid composition of porcine backfat.[1]

	Experiment			
	1		2	
	Backfat layer		Backfat layer	
Fatty acid:[2]	Inner[3]	Outer	Inner	Outer
14:0, %	1.1 ± 0.2	1.2 ± 0.2	0.8 ± 0.2	0.9 ± 0.2
16:0, %	22.8 ± 0.3	22.7 ± 0.3	19.8 ± 0.6	19.2 ± 0.6
16:1, %	3.4 ± 0.2[a]	4.1 ± 0.2[b]	3.5 ± 0.4[a]	3.7 ± 0.5[b]
18:0, %	17.9 ± 0.4[b]	13.7 ± 0.3[a]	16.8 ± 0.3[b]	12.9 ± 0.3[a]
18:1, %	45.9 ± 0.7[a]	47.8 ± 0.8[b]	45.1 ± 0.7[a]	47.5 ± 0.8[b]
18:2, %	9.0 ± 0.4[a]	10.5 ± 0.4[b]	13.9 ± 0.2[a]	15.8 ± 0.4[b]
Total saturated, %	41.7 ± 0.8[b]	37.6 ± 0.7[a]	37.4 ± 0.8[b]	33.0 ± 0.7[a]

[1] All means with the same superscripts or those without superscripts are not significantly different ($P > .05$) within each experiment.
[2] Carbon chain length:number of double bonds. Fatty acids are expressed as % of total methyl esters.
[3] Mean ± standard of error of the mean.

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A Study of Certain Properties of Myosin from Skeletal Muscle

SUMMARY—This investigation was conducted (a) to establish a procedure for purifying a stable myosin preparation from pig skeletal muscle and (b) to evaluate the enzymatic activity and associated characteristics of purified myosin, isolated at death from skeletal muscles which ultimately have varying rates of hydrolysis of ATP *in situ* during the first half-hour after death. Rabbit muscles were also used for comparative purposes. Myosin preparations were found to be pure (by ultracentrifugation, Sephadex separation and superprecipitation tests) and stable with normal values for SH groups. The Ca^{++}-activated ATPase activities of myosin extracted from PSE Poland China pigs were significantly greater than those from Chester White pigs and normal Poland China pigs. EDTA-activated ATPase activities were greater in myosin from PSE Poland China than in normal Poland China.

INTRODUCTION

THE PRIMARY BIOCHEMICAL characteristic of myosin is its enzymatic activity with respect to the hydrolysis of ATP (Engelhardt *et al.*, 1939). The ATPase activity of purified myosin is related to the ATPase activity of reconstituted actomyosin (Barany, 1967) and to the interaction between actomyosin and ATP *in vivo*, (i.e., muscular contraction, Mommaerts, 1950, 1966; Davies, 1963).

Substantial differences have been reported in the ATPase activity of myosin extracted from the skeletal muscle of various species (Bailey, 1942; Perry, 1960). In addition, as an animal grows, there is a concurrent progressive increase in its myosin ATPase activity (Trayer *et al.*, 1966; Perry *et al.*, 1963; Barany *et al.*, 1965a; Khyl'ko, 1965). Finally, several workers have recently demonstrated that, within the same animal, myosin extracted from white muscle has higher ATPase activity than that from red muscle (Barany *et al.*, 1965b; Sreter *et al.*, 1966; Seidel *et al.*, 1964). These findings substantiate the suggestion (Perry, 1960) that myosin is an adaptive enzyme.

An increased rate of hydrolysis of ATP during the first half-hour after death in the muscles of the pig (Briskey *et al.*, 1961; Bendall *et al.*, 1962) has been related to the development of the pale, soft, exudative (PSE) condition (Briskey, 1964), but the relationship of the enzymatic activity of myosin *in vitro* to this increased ATPase postmortem *in situ* and the consequent PSE condition has not been established.

This preliminary investigation was conducted: (a) to establish a procedure for purifying a stable myosin preparation from pig skeletal muscle which is high in lipid content, (lipids are known to affect myosin stability and ease of purification) (Lynn, 1965; King *et al.*, 1962), and (b) to evaluate the enzymatic activity and associated characteristics of purified myosin, isolated at death from skeletal muscles which subsequently have varying rates of hydrolysis of ATP *in situ*.

The muscle used was the longissimus dorsi from Chester White and Poland China pigs, of which the latter were divided into two groups depending on whether or not they developed the PSE condtion. Since most of the previously reported studies on purified myosin have been performed using rabbit muscle, preparations from the longissimus dorsi of this species were also evaluated for comparison purposes.

MATERIALS AND METHODS

Muscle source

At the time of death the longissimus dorsi muscles from five or six animals in each of the following four groups were used in this study: (a) Chester White pigs which ultimately had normal muscle characteristics post-mortem (slow ATPase activity *in situ* soon after death), (b) Poland China pigs which also ultimately had normal muscle characteristics post-mortem, (c) Poland China pigs which ultimately had PSE muscle characteristics postmortem (rapid ATPase activity *in situ* soon after death), and (d) New Zealand white rabbits. The rabbits and pigs were immobilized and exsanguinated directly or injected,

respectively, with sodium pentobarbital (90 mg) and α-turbocurarine chloride (1.5 mg) or nembutal and Dial with urethane (50/50) (0.3 ml/kg) prior to exsanguination.

Myosin preparation

Myosin was prepared according to the flow diagram outlined in Fig. 1. The procedure, a modification of the early procedures of Szent-Gyorgyi (1942) and Mommaerts (1950), utilized some of the changes adapted by Azuma *et al.* (1965) and by Seraydarian *et al.* (1967a) and was otherwise modified to fit our experimental conditions. Special attention should be directed toward certain details of the procedure.

Step IV, the filtration through glass-wool, was included because of the copious quantities of lipid material in myosin extracts from pig muscle. The myosin was protected from the adverse effects of glass-surfaces by first washing the glass-wool twice with EDTA to remove heavy metals, then with deionized water several times to remove the EDTA, and finally with 0.5*M* KCl. This was the only step in the procedure in which glass-surfaces were used; all beakers, centrifuge tubes, etc. were of plastic composition.

Allowing the solution to remain undisturbed for 20 min at 0.27*M* KCl, in Steps V and VII, enhanced the complete precipitation of contaminating actin as actomyosin (Azuma *et al.*, 1965). Precipitation of the myosin, in Steps II, VI and VIII, was initiated by diluting the solution slowly to 0.06*M* KCl. This precipitation was then enhanced by stirring slowly for about 2 min before diluting further to 0.03*M* KCl.

Washing the collected myosin precipitate with 0.03*M* KCl in Steps III, VII and IX, provided some advantages which may be of only minor importance. The volume of the collected, washed precipitate could be measured more accurately, and this knowledge along with a knowledge of the KCl concentration of the gel provided a finer control of the KCl concentration in subsequent steps. Knowledge of the volume of the precipitate also provided rough estimates of the yields at these steps in the procedure. Finally, this precautionary wash provided further opportunity for the removal of contaminating water-soluble proteins, nucleoproteins or free nucleotides, buffers and lipids.

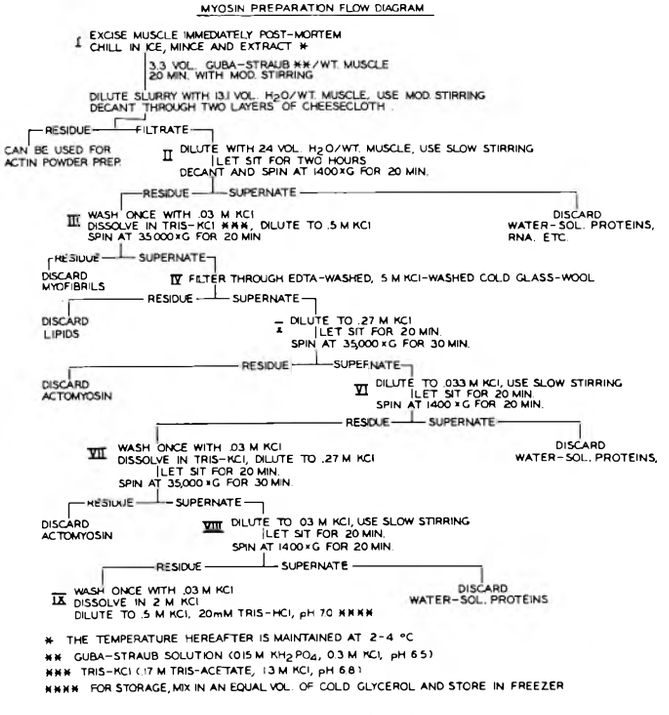

Fig. 1. Myosin preparation flow diagram.

The purified myosin preparations, adjusted to 0.5*M* KCl, 20 m*M* Tris-HCl, pH 7.0, at a concentration of about 40 mg/ml, were analyzed within five days or stored in glycerol at −20°C. The temperature of all solutions and equipment was maintained at 4°C throughout the procedure except during centrifugation when the rotor chamber was maintained at 0°C.

Estimations of purity

Ultracentrifugation. The ultracentrifugation was performed using a Spinco Model E Analytical Ultracentrifuge equipped with electronic speed control, automatic temperature control and adjustable optical components. The AN-D rotor, operated at 60,000 RPM and 20°C, contained two 12 mm cells with Kel-F centerpieces, one having a plain quartz upper window, the other having a 1° positive wedge quartz upper window. The sequence of schlieren patterns was obtained from myosin samples diluted to 2 mg/ml in 0.5*M* KCl, 20 m*M* Tris-HCl, pH 7.0, with automatic 16 min photographic sequence and 3 sec exposure.

Sephadex G-200 separation. The Sephadex G-200 was purchased from Pharmacia (Lot No. 7367) and prepared for column packing by allowing to hydrate in excess deionized water for three days at room temperature. The small analytical columns (1 × 28 cm) were packed at room temperature, then equilibrated at 4°C with 0.5*M* KCl, 20 m*M* Tris, pH 7.0. Elution of the sample, with the equilibrating buffer, was by gravity flow (6–12 ml/hr) using a hydrostatic head which did not exceed 15 cm. The elution patterns were obtained after application of 10–20 mg of myosin in 1–2 ml by measuring the optical density of the collected fractions at 280 mμ and at 250 mμ.

Extinction coefficients. The extinction coefficients at 280 mμ and 250 mμ were calculated by dividing the optical density of the myosin solution by the protein concentration of that solution. The myosin solutions were diluted to 0.5 mg/ml in 0.5*M* KCl, 20 m*M* Tris, pH 7.0. The optical density at 280 mμ and at 250 mμ was determined in a 1 cm cell in a Beckman DB Spectrophotometer using the solvent as a reference. The protein concentration was determined using a biuret procedure. The ratio of the extinction coefficients, "R", was calculated by dividing the optical density at 250 mμ by the optical density at 280 mμ.

Estimation of protein concentration

The concentration of protein in the diluted myosin solutions was determined by the Gornall *et al.* (1949) biuret procedure. Crystallized bovine serum albumin was used as the standard and 0.5*M* KCl, 20 m*M* Tris, pH 7.0 as the blank. The optical density at 540 mμ was measured in a 1 or 4 cm cell in a Beckman DB Spectrophotometer using biuret plus water as the reference.

Estimation of sulfhydryl group content

The method used for estimating the free SH content of the myosin preparations was Boyer's (1954) pCMB titration as modified by Sela *et al.* (1959) and further modified by Seraydarian *et al.* (1967b). This procedure involved titration of the free, readily reactive SH groups with a mercurial, pCMB, to form a mercaptide. The concentration of the titrating solution, which was diluted to about 0.07 m*M* pCMB, was determined spectrophotometrically. (The molar extinction coefficient at 232 mμ of neutralized pCMB is 16.9 × 10^6 cm^2/mole, Boyer, 1954.) The concentration of the myosin to be titrated, which was diluted to 0.5 mg/ml, was determined by the aforementioned biuret procedure.

After allowing the protein and pCMB to react at room temperature, for at least 7 hr, the optical density of each tube at 255 mμ was measured in a Beckman DB Spectrophotometer using H_2O as the reference. The intersection of the two straight lines obtained by plotting for each tube; the optical density at 255 mμ versus the ml of pCMB titrating solution added was the equivalence point and was related to the number of free SH groups per unit weight of protein (Fig. 2).

ATPase activity

The time course of the hydrolysis of ATP (media described in captions to appropriate figures) at 30°C was followed by taking aliquots at four time intervals and estimating the amount of inorganic phosphate produced using the method of Fiske *et al.* (1925). The myosin was diluted to a concentration of 2 mg/ml for the Ca^{++}-activated ATPase and 0.5 mg/ml for the EDTA-activated ATPase. One ml of the respective enzyme solution was used in a total reaction mixture of ten ml. The reaction was started by adding the ATP (within one minute after adding the enzyme) and stopped by mixing a 2 ml aliquot with 1 ml of cold 15% TCA.

Following centrifugation, 2 ml of the supernate were added to 2.3 ml of H_2O and 0.5 ml of 2.5% ammonium molybdate in 5*N* H_2SO_4. Immediately thereafter, 0.2 ml of Fiske-SubbaRow reducer were added and the color allowed to develop for 10 min. It was found that reproducible results could best be obtained by holding in a 37°C water bath during this color development period. The optical density at 660 mμ was measured in a 1 cm cell in a Beckman DB Spectrophotometer using H_2O as a reference. A solution of KH_2PO_4 was used for standards and a solution containing 1 m*M* ATP only used as a blank. The slope of the line obtained by plotting m*M* Pi in the reaction mixture versus time was used to calculate the enzymatic activity.

The myosin ATPase activity was determined both in the presence of the activator during a 3 min total reaction time and in the absence of the activator during a 30 min total reaction time.

RESULTS AND DISCUSSION

The difficulties involved in purifying myosin extracted from pig muscle, which contains large quantities of lipid material, have been resolved without using extremely rigorous or time-consuming steps (see Fig. 1). The entire procedure can be completed in a single day. Most of the lipid contamination in the initial myosin extract was removed by filtering through glass-wool, and the slight lipid film which was still visible following Step V dis-

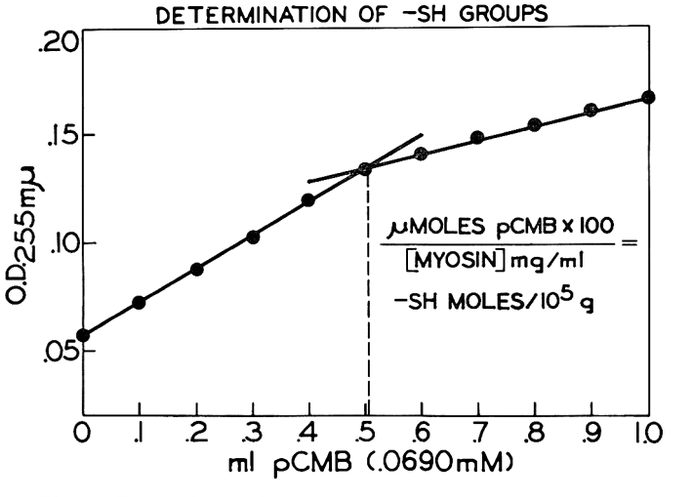

*Fig. 2. Estimation of SH groups in myosin by pCMB titration: 1.0 ml of myosin at 0.5 mg/ml in 0.5*M *KCl, 20 m*M *Tris, pH 7.0; 1.0 ml of buffer (1.5*M *KCl, 65 m*M *Tris, pH 7.5); 0–1.0 ml of 0.07 m*M *pCMB; 1.0–0 ml of* H_2O.

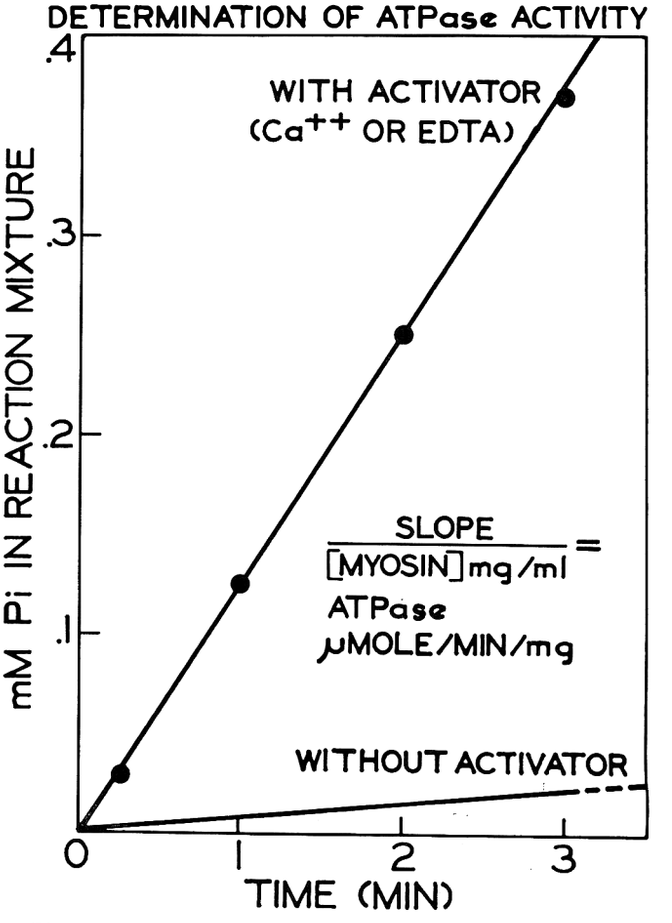

Fig. 3. Determination of ATPase activity of myosin at 30°C: 1.0 ml of myosin in a 10 ml reaction mixture; 2.0 ml aliquots taken at 4 time intervals.

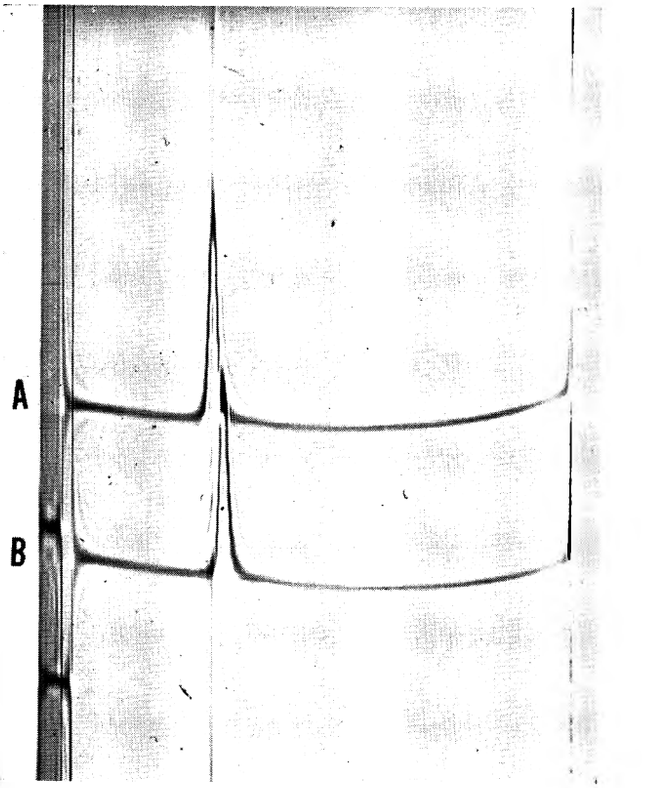

Fig. 4. Schlieren pattern of purified myosin isolated from pig muscle: A. Chester White myosin (1.99 mg/ml); B. PSE Poland China myosin (1.90 mg/ml). Protein is in 0.5M KCl, 20 mM Tris, pH 7.0 60,000 rpm, 20°C, 56 min after 2/3 speed.

appeared with further purification just as it did with myosin purified from rabbit muscle.

The techniques used to estimate the purity of these myosin preparations indicated that myosin of a high degree of purity was isolated from each of the muscle sources. These preparations contained very little lipid or nucleotide contamination, appeared homogeneous in the ultracentrifuge (Fig. 4) (Schachman, 1959), and showed no detectable actin contamination by the superprecipitation test of Azuma *et al.* (1965). (This test will demonstrate the presence of as little as three parts of actin per 1000 parts of myosin.)

Furthermore, preliminary storage experiments on these preparations have revealed adequate stability at 0°C for 2 weeks at a concentration of 20 mg/ml in 0.5*M* KCl, 20 m*M* Tris, pH 7.0, and excellent stability in 50% glycerol at −20°C for several weeks. However, after two weeks, the SH content began to decrease and a visible dimer appeared in the schlieren patterns in the ultracentrifuge (Fig. 5). After eight weeks, the SH content was decreased to about 5 moles/10^5 g and the EDTA-activated ATPase activity decreased along with the decrease in SH content. Storage in 50% glycerol at −20°C retarded these deteriorations (Fig. 5).

It has been previously reported (Richards *et al.*, 1967) and was confirmed in this report that myosin dimers can be separated from the monomers by filtration through an inverse molecular sieve (i.e. Sephadex). Small analytical columns were used in this study for the purpose of expediency. Myosin was eluted from the column after one void volume (Fig. 6) with the dimer being concentrated in the leading edge of the peak (Fig. 5).

Myosin from these preparations, when eluted from the columns, did not reveal the large trailing shoulder which was observed in other reports (Richards *et al.*, 1967; Smoller *et al.*, 1964). This trailing shoulder would contain nucleoproteins or myosin bound to contaminating nucleotides, as well as some free nucleotides. The small amount of trailing material which was obtained from these preparations had a maximum absorbance at 260 mμ which indicated that a slight contamination of this nature was present and was eluted in the expected manner. This fraction was anticipated by the large increase in the ratio of the optical densities ($R = OD_{250}/OD_{280}$) as seen in Fig. 6.

Note the change in "R" within the leading edge of the peak. The fraction which demonstrated the presence of a dimer in the ultracentrifuge had a slightly greater "R" than that freed of the dimer (determined after diluting both to about 0.5 mg/ml since there was a slight concentration dependence). This difference in "R" was even greater when one compared the main fraction with the leading edge which would contain an even higher proportion of dimer. The ratio in this leading edge was also greater than that of the sample (diluted) before application to the column.

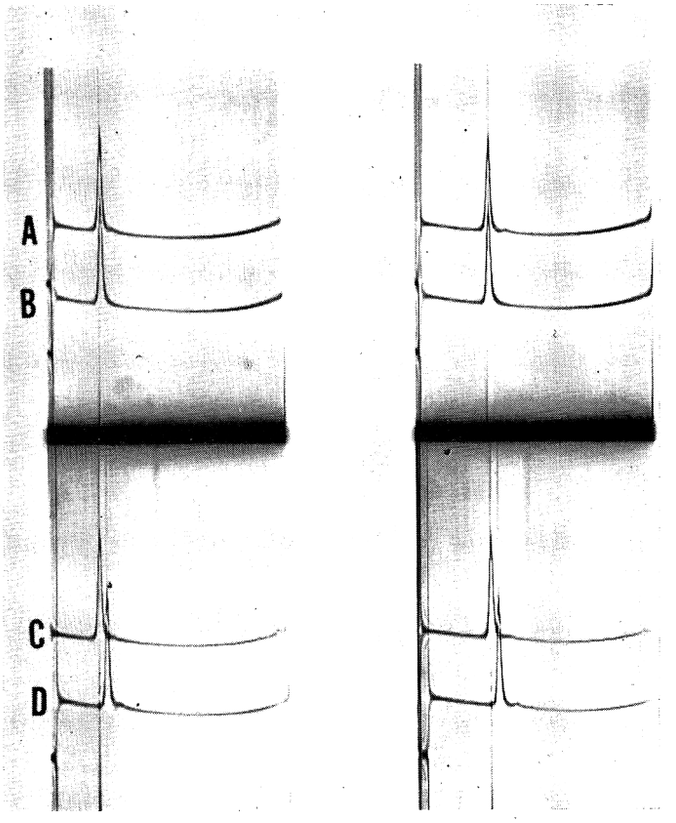

Fig. 5. Schlieren patterns of purified pig myosin: A. PSE Poland China myosin (2.05 mg/ml) aged 2 weeks at 0°C; B. Rabbit myosin (2.05 mg/ml) stored in 50% glycerol at −20°C. 38 and 54 min after 2/3 speed; C. Fraction No. 3 from a Sephadex G-200 column (2.41 mg/ml, see also Fig. 6); D. Fraction No. 2 from a Sephadex G-200 column (2.40 mg/ml, see also Fig. 6). 42 min and 58 min after 2/3 speed 60,000 rpm, 20°C, in 0.5M KCl, 20 mM Tris, pH 7.0.

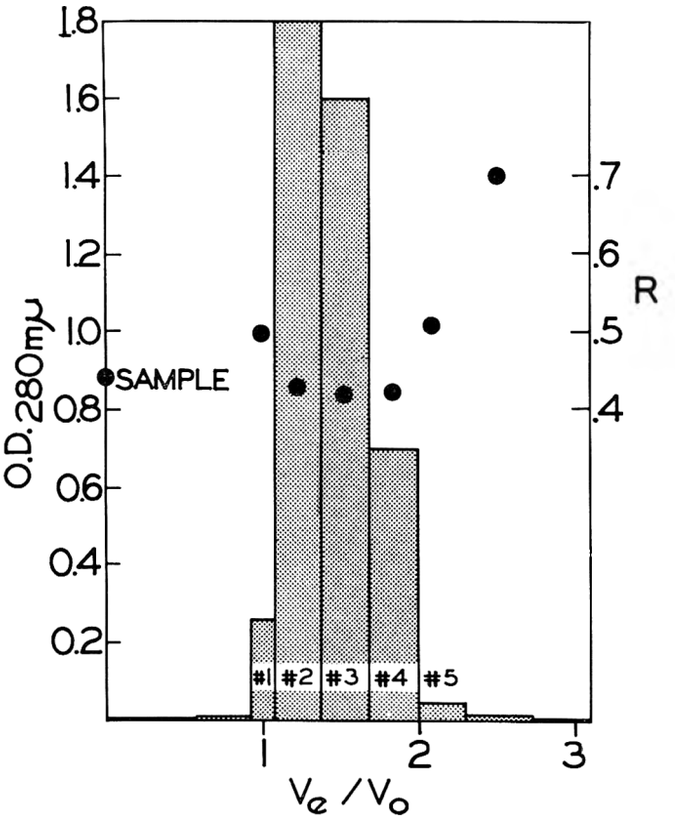

Fig. 6. Elution pattern of myosin filtered through a 1 × 28 in. Sephadex G-200 column at 6–12 ml/hr, 4°C. Sample size is 1 ml of 15 mg/ml in 0.5 M KCl, 20 mM Tris, pH 7.0. Ve = elution volume, Vo = void volume 8 ml, R = OD250/OD280.

Preliminary storage experiments demonstrated that "R" increased with duration of storage and that this increase was accompanied by the appearance of a dimer in the schlieren patterns from the ultracentrifuge, and by the decrease in SH content and EDTA-activated ATPase activity as has already been mentioned. However, it remains to be demonstrated that the dimers have a lower SH content than the monomers from which they were separated, in order to ascertain whether dimerization occurs through intermolecular disulfide formation. It may be that fatty acids or nucleotides provide these intermolecular cross-links.

It appears that R, the ratio of the OD_{250} to the OD_{280}, may be used as an estimate of purity both with respect to nucleotide contamination and with respect to the formation of dimers.

A scan of the absorbance of myosin dissolved in 0.5*M* KCl, 20 m*M* Tris, pH 7.0, using the solvent as the reference, showed a maxima at 280 mμ and a minima at 250 mμ which led to the determination of the extinction coefficients at these two wavelengths (see Long, 1961). The average extinction coefficient (Fig. 7) at 280 mμ ($E^{1\%}_{280}$ = 5.2) for rabbit myosin was lower than most values reported in the literature (Trayer *et al.*, 1966; Milhalyi *et al.*, 1966; Gellert *et al.*, 1963; Woods *et al.*, 1963; Kielley *et al.*, 1960) and was closer to the theoretical value of 4.90 (Kielley *et al.*, 1960) based on the amino acid composition of rabbit myosin.

The similarity in $E^{1\%}_{280}$ between rabbit myosin and

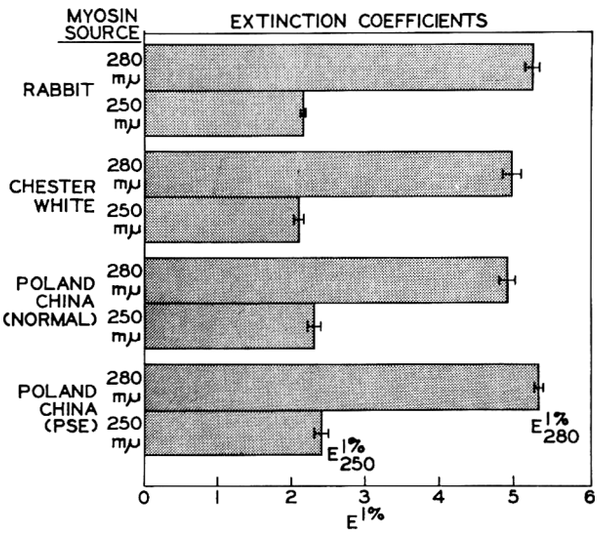

Fig. 7. Extinction coefficients determined at 250 mM and at 280 mM at a concentration of 0.5 mg/ml in 0.5M KCl, 20 mM Tris pH 7.0. $E^{1\%}$ represents the absorbance if the solutions were 10 mg /ml (1%).

PSE Poland China myosin indicated a possible similarity in amino acid composition. The average $E^{1\%}_{280}$ for normal Poland China myosin and Chester White myosin were not significantly less than that for PSE Poland China myosin and were even closer to the theoretical value already mentioned. The extinction coefficients have not been corrected for light scattering (Milhalyi *et al.*, 1966) ($E^{1\%}_{320}$ < 0.02 in most samples).

The ratio of the maxima and minima (R = OD_{250}/OD_{280}) for these purified preparations can be converted to the more commonly reported ratio of OD_{260}/OD_{280} by multiplying by 1.4. This factor was the ratio of OD_{260}/OD_{280} and it varied somewhat depending on the purity of the sample. The average ratio of the extinction coefficients (or optical densities reported here for rabbit myosin) (R = 0.42) was much lower than corresponding values (after adjusting to OD_{250}/OD_{280}) previously reported in the literature (Trayer *et al.*, 1966; Woods *et al.*, 1963;

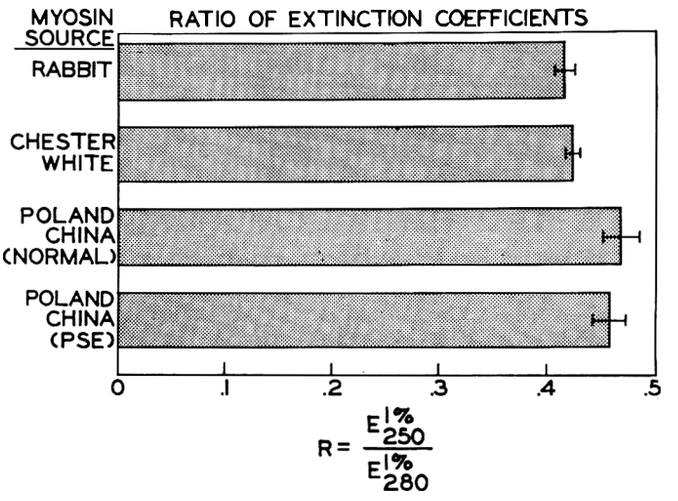

Fig. 8. Ratio of extinction coefficients.

Baril *et al.*, 1964, 1966; Smoller *et al.*, 1964) unless more time consuming purification steps were taken, such as chromatography through DEAE-cellulose.

The pCMB titration of myosin SH groups was not a measure of the total sulfhydryl content, but rather an estimate of the free reactive sulfhydryl groups (those reacting with a mercurial in less than seven hr at room temperature, pH 7.5, .67*M* KCl, 33 m*M* Tris). The relationship between myosin SH groups and myosin ATPase activity has been investigated by many workers (Gergely, 1966) for several years and the following two generalizations have emerged: Reaction of the most reactive SH group with a sulfhydryl reagent increases the Ca^{++}-activated ATPase activity and decreases the EDTA-activated ATPase activity. Further reaction with sulfhydryl reagents reduces the EDTA-activated ATPase activity to zero and reverses the effect of lower concentrations of sulfhydryl reagents on Ca^{++}-activated ATPase activity.

The estimated moles of SH groups per 10^5 g of purified myosin extracted from rabbit skeletal muscle (Fig. 9) had a range of 6.6 to 7.2 and an average of 6.8 which was similar to the 7 moles SH/10^5 g reported by previous workers using the same method (Seraydarian *et al.*, 1967b). The average SH content of myosin from the PSE Poland China pigs agreed very well with that for rabbit myosin which indicated that myosin could be prepared, without significant loss of labile SH groups, from pig muscles. Furthermore, using pig muscle, myosin prepared in the presence of DTT (which serves to prevent the oxidation of SH groups, Cleland, 1964) was not significantly higher in SH content than that prepared according to the standard procedure.

Myosin prepared from Chester White pigs and normal Poland China pigs had a slightly lower average SH content than that from PSE Poland China pigs; however, this difference was not significant. Myosin from all four muscle sources appeared to have comparable SH content.

The specific enzymatic activity of the myosin prepared from each muscle source was determined both in the presence and absence of the activators (1 m*M* Ca^{++} or 1 m*M* EDTA). The original intent in determining the activity in the absence of the activator was to correct for subtle differences between muscle sources by subtracting the

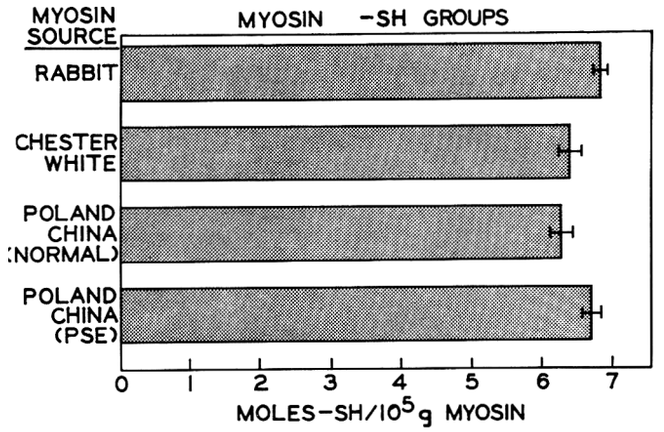

Fig. 9. Myosin SH groups—mean and standard error of the mean.

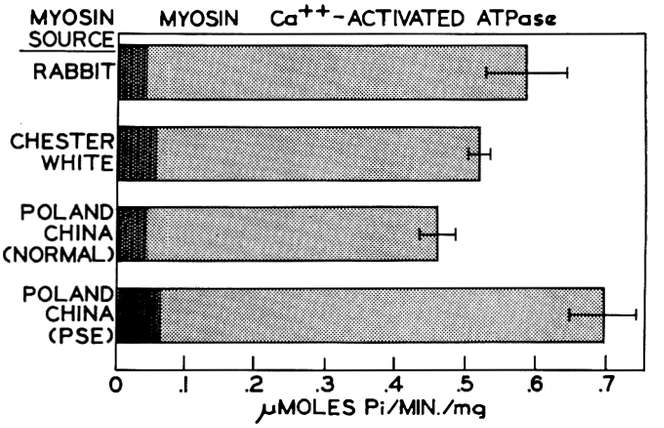

Fig. 10. Myosin Ca^{++}-activated ATPase activity—mean and standard error of the mean. Conditions: ~ 0.20 mg/ml, (1 mM Ca^{++}) 1mM ATP, 50 mM KCl, 20 mM Tris, pH 7.0, 30°C.*
** With and without the activator.*

latent ATPase activity (determined in the absence of the activator) from the total activated ATPase activity (determined in the presence of the activator) in order to obtain the true activated ATPase, that is, the increase in activity due to the addition of the activator. However, as can be seen in both Fig. 10 and 11, the latent ATPase does not differ sufficiently between breeds or between species to alter the significance of the comparison of the uncorrected total activated ATPase activity.

The effect of two activators, Ca^{++} and EDTA, were both investigated because of the opposite effect of a small amount of sulfhydryl reagent on the ATPase activity determined in the presence of these two activators, as has already been mentioned.

The Ca^{++}-activated ATPase activity (Fig. 10) determined for rabbit myosin agreed very well with that reported by previous investigators if adjustments were made for the differences in assay conditions. The Ca^{++}-activated ATPase activity of myosin extracted from PSE Poland China pigs was not significantly greater than that from rabbits at the 5% level, but was significantly greater than that from Chester White pigs and normal Poland China

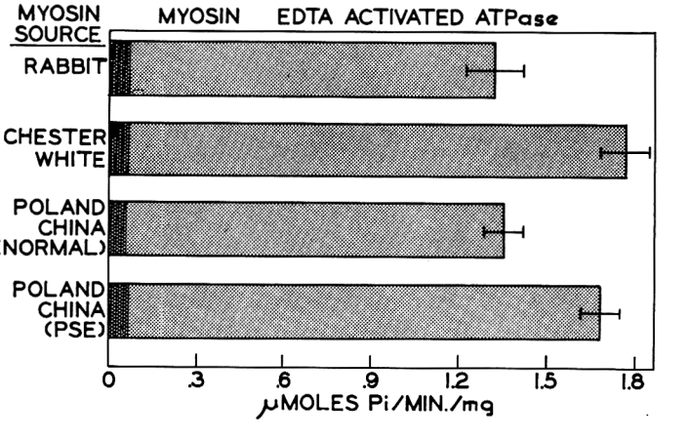

Fig. 11. Myosin EDTA-activated ATPase—mean and standard error of the mean. Conditions: ~ 0.05 mg/ml, (1 mM EDTA), 1 mM ATP, 0.5M KCl, 20 mM Tris, pH 7.0, 30°C.*
** With and without the activator.*

SOURCE	-SH MOLES /10^5g	ATPase μMOLES/MIN/mg Ca^{++}-ACTIVATED		EDTA-ACTIVATED	
RABBIT MEAN	6.8	.59	-.04 = .55	1.33	-.23 = 1.10
RABBIT $S_{\bar{x}}$	.1	.06		.10	
CHESTER WHITE	6.4	.52	-.06 = .46	1.77	-.22 = 1.55
	.2	.02		.08	
POLAND CHINA NORMAL	6.3	.46	-.04 = .42	1.36	-.19 = 1.17
	.2	.03		.07	
POLAND CHINA PSE	6.7	.70	.06 = .64	1.68	-.23 = 1.45
	.1	.05		.07	

Fig. 12. Summary.

pigs. The difference between normal and PSE Poland China pigs was significant at the 1% level.

The EDTA-activated ATPase activity (Fig. 11) determined for rabbit myosin corresponded to the white muscle myosin studied by Sreter *et al.* (1966). Some similarities can be seen in the pattern of activities in the two assay mediums; namely, myosin from PSE Poland China pigs had greater activity than myosin from rabbits. Also, myosin from PSE Poland China pigs had greater ($P < .05$) activity than myosin from normal Poland China pigs.

However, some dissimilarities were apparent; the activity of myosin from Chester White pigs was equal to that of myosin from PSE Poland China pigs.

The enzymatic properties studied in this preliminary investigation were selected to examine the possible relationship between the rate of ATP hydrolysis soon after death in the intact tissue and the enzymatic activity of the purified myosin isolated at death from that tissue. Data for myosin from animals of the same species and the same breed but with different rates of ATP hydrolysis soon after death (i.e., normal and PSE Poland China pigs) indicated a slightly higher SH content and significantly greater Ca^{++}-activated and EDTA-activated ATPase activity in the myosin from muscles which ultimately became PSE than from those which retained normal characteristics. There does seem to be some direct correlation between the enzymatic activity of myosin *in vitro* and the ATP splitting in the muscle *in situ*.

It is possible that the increased Ca^{++}-activated ATPase activity, in the myosin from the PSE pigs, could be a result of a reaction between the myosin and a low concentration of a substance which reacts with sulfhydryl groups. A possible substance could be a denatured sarcoplasmic protein (such as creatine kinase). However, subsequent to such a reaction, the sulfhydryl content would be slightly reduced and the EDTA-activated ATPase activity would also be reduced. Since neither the sulfhydryl content or EDTA-activated ATPase have been altered, it is not likely that such a derivative has been formed in these studies, or at least, it has not survived the purification procedures.

As an animal grows, its myosin SH content increases (Khyl'ko, 1965), and its myosin Ca^{++}-activated (Trayer *et al.*, 1966; Barany *et al.*, 1965a; Khyl'ko, 1965; Perry *et al.*, 1963) and EDTA-activated ATPase activity increases. This leads to speculation that the longissimus dorsi muscles in normal Poland China pigs have not developed to the same extent as in the PSE Poland China pigs. This same line of reasoning, with consideration of the data for myosin from animals of the same species and with similar rates of ATP hydrolysis soon after death but of different breeds (i.e., normal Poland China pigs and Chester White pigs), would lead one to speculate that normal Poland China pigs have not developed to the same extent as Chester White pigs.

Perhaps the low pH-high temperature condition which is characteristic of muscle which ultimately becomes PSE, further aggravates an inherent problem by denaturing the sarcoplasmic reticulum and causing the release of greater quantities of free Ca^{++}, which, in turn, further increase the rate of ATP hydrolysis (Greaser *et al.*, 1968). Additional studies are required to further establish the direct association of the characteristics of the isolated proteins with *in situ* muscle behavior post-mortem.

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Composition of Bovine Muscle Lipids at Various Carcass Locations

SUMMARY—Bovine intramuscular lipids extracted from the semitendinosus, triceps brachii and longissimus dorsi muscles were fractionated into phospholipids and neutral fats by silicic acid column chromatography. In spite of the wide range in total fat content at each location, phospholipids were present in all three muscles at a level of approximately 500 mg per 100 g of muscle tissue. This result, coupled with the lower total fat content of the semitendinosus as compared to the other two muscles, indicated a significantly higher percentage of phospholipid material in the total fat from the semitendinosus as compared to the triceps brachii or longissimus dorsi.

The fatty acids were identified in both lipid fractions using retention time data obtained on both a polar and a non-polar column. The identity of the unsaturated fatty acids was confirmed when their peaks did not appear on the chromatographs obtained from brominated samples. There was significantly more C14:0 in the longissimus dorsi neutral fat fractions than in the semitendinosus neutral fat fractions. In the phospholipids, there was significantly more C16:0 and significantly less C18:0 in the longissimus dorsi as compared to either the semitendinosus or triceps brachii. Although the two lipid fractions of the longissimus dorsi contained slightly higher percentages of total saturated fatty acids than the corresponding fractions in the other two muscles, the effects were not significant.

INTRODUCTION

ALTHOUGH MUCH WORK has been done on the composition of the depot fats of large meat animals, the intramuscular lipids have not been studied to the same extent. There is an unfortunate lack of knowledge when consideration is given to the factors involved in determining the stability of muscle tissue lipids towards oxidative rancidity.

Intramuscular lipids, unlike depot fats, contain significant amounts of phospholipid material. Hartman *et al.* (1957) found that the intramuscular lipids of mutton loin chops contained a higher percentage of C20 unsaturated fatty acids than the adipose tissue fat. This was attributed to the presence of 5–8% of phospholipid material in the intramuscular fat which had no counterpart in the adipose tissue fat.

That the phopholipids are a rich source of polyunsaturated fatty acids was also noted by Ostrander *et al.* (1962) during the course of a study on the composition of the covering fat, intramuscular fat and intermuscular fat of beef, pork and lamb. Hornstein *et al.* (1961) undertook a more detailed investigation of the fatty acid composition of the phospholipid and neutral fat fractions of beef and pork muscle. Over 50% of the fatty acids in the phospholipids contained two or more double bonds compared to 10% in the case of the neutral fat. It was also found by these workers that the phospholipids developed rancid off-flavors much more readily than the neutral fats on exposure to the atmosphere. The instability of phospholipids in the presence of atmospheric oxygen had previously been observed by Younathan *et al.* (1960) during the course of a study on the oxidation of cooked pork.

It appears, therefore, that the phospholipids are of some importance in determining the stability of intramuscular lipids toward oxidative rancidity. However, very little information is available on the composition of phospholipids in different mammalian muscle tissues. Kuchmak *et al.* (1963) noted that there were differences due to location in the fractional composition of the phospholipids in the hog carcass. Hidaka *et al.* (1965) found that muscle location had no significant effect on the fatty acid composition of neutral fat and phospholipids in the beef carcass.

This study was conducted to determine the relative amounts of phospholipids and neutral fats at different locations in the beef carcass and to further analyze these lipid fractions for their fatty acid composition.

EXPERIMENTAL

Five hundred g samples were taken from the semitendinosus, the longissimus dorsi and the triceps brachii muscles of eight Hereford, three Angus and two crossed Angus-Charolais beef animals. Two of the Hereford cattle and one of the Angus cattle were heifers; the remaining animals were steers.

After trimming off all external fat, the samples were placed in No. 2 lacquered tin cans and the end seams were sealed with a silicone rubber preparation (RTV adhesive, General Electric). A small hole was punctured in one end of each can. The cans were placed in a vacuum desiccator which was then evacuated and finally the vacuum was broken with nitrogen gas. The evacuation procedure was repeated a total of three times and the holes on the cans were then sealed with solder.

The cans were stored in a −20°C freezer until they were required for further analysis.

Extraction of muscle lipids

Samples of muscle tissue were removed from storage as needed and allowed to thaw at room temperature. After taking a sample out of a can, the meat was cut into small cubes and 100 g were added to a Waring blendor. The muscle tissue was then homogenized with a mixture of chloroform, methanol and water. The extraction procedure was essentially that of Bligh *et al.* (1959) as modified by Ostrander *et al.* (1961). After the extraction had been completed, the chloroform phase was separated from the aqueous phase in a 1-L separatory funnel and dried over anhydrous sodium sulfate.

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The solvent was removed on a "Rinco" rotary evaporator, initially under partial vacuum, and the contents were then transferred to a tared 150 ml flat-bottomed flask. The last traces of solvent were removed under high vacuum using a positive displacement vacuum pump in conjunction with a dry-ice ethanol trap. The flask was placed in a vacuum desiccator containing potassium hydroxide pellets, until it reached constant weight.

Fractionation of muscle lipids

The total intramuscular lipids were separated into neutral fats, pigmented material and phospholipids on a 2.5 × 90 cm glass column containing 50 g of silicic acid using the procedure of El-Gharbawi *et al.* (1965). The fractions were concentrated and dried by the techniques described previously for the total fat extraction. The pigmented material, accounting for less than 1% of the total lipids and consisting mostly of carbohydrates, was discarded after being taken to constant weight.

Esterification

The method of DeMann (1964) was modified for use with intramuscular lipids. The capillary tube of a 5.75 inch disposable pipet was filled to half of its capacity with fat and this material was then added to a 1 ml ampoule. By means of a Luer syringe of 1 ml capacity, 0.75 ml of 0.05*N* sodium methoxide was delivered into the ampoule. The absolute amounts of sodium methoxide and fat were not critical if the ratio of sodium methoxide to fat was at least 3:1 (v/v).

After sealing the ampoule, the samples were transferred to a constant temperature water bath at 60°C. Completion of the reaction was apparent when the sample mixture changed from a two phase system to a single phase system. This generally occurred after 1.5 hr with the neutral fats but 2.5 hr were required where phospholipid material was being esterified.

After removal from the water bath, the sample was cooled to room temperature and then stored at −20°C until required for further analysis.

The lipid fractions from 7 of the 13 animals were converted to their fatty acid methyl esters.

Gas chromatography

Initially several samples of methyl esters were analyzed by the dual column chromatographic technique described by Vorbeck *et al.* (1961, 1963). The non-polar hydrocarbon, Apiezon L, was used as the substrate in one column and the polar polyester, diethylene glycol succinate, served as the partitioning medium in the second column. A mixture of standard fatty acid methyl esters was separated on both of the columns and the $\log_{10}$ retention times relative to palmitic acid were calculated. When the values obtained from the non-polar column were plotted against those obtained on the polar column, a grid was formed as described by James (1959). Using this grid it was possible to tentatively identify unknown fatty acids in those cases where standards were not available.

To further establish the identity of some of the unknown unsaturated fatty acids, the samples were brominated by a modification of the method of James *et al.* (1956) using ethanolic bromine in place of an ethereal solution of bromine. The chromatographic results from the original samples and the brominated samples were then compared to determine which peaks had been eliminated following bromination.

Quantitative analysis

An F & M Model 609 gas chromatograph equipped with an F & M Model 400 flame ionization detector was used to obtain quantitative data on the fatty acid composition of the samples. The column consisted of a glass U tube, 4 mm I.D. and 6 ft long. The stationary phase was 15% diethylene glycol succinate coated on Chromasorb W, 80–100 mesh (Applied Science Laboratories, Inc., College Park, Penn.).

After the column had been uniformly packed with the aid of a vibratory tool, it was preconditioned by baking for 24 hr at a column temperature of 160°C and a helium flow rate of 60 ml per min. At the end of this period of time the baseline drift and noise level were minimal.

The column temperature was equilibrated to 155°C prior to injection of the sample. As the injection port was directly on the column, the temperature was maintained the same. The temperature in the detection cell was approximately 180°C with an air flow rate of 400 ml per min and a hydrogen flow rate of 65 ml per min. All gas tank outlet pressures were adjusted to 40 psi.

A 1-μl aliquot was taken directly from the ampoule containing the sample of methyl esters and injected on the column. This proved adequate for the determination of fatty acid methyl esters present at 0.1% or more of the total methyl esters.

The peak areas on the chromatogram were evaluated by triangulation (½ base × height). The weight percentage of each fatty acid in a sample was determined by expressing its peak area as a percentage of the total peak area. Replicates were run on all samples.

Determinations of the significance of the correlation coefficients and calculations for Tukey's test for the comparison of means were carried out according to Steel *et al.* (1960).

RESULTS AND DISCUSSION

Fractionation of beef intramuscular lipids

The results of the initial phase of the study involving fractionation of the intramuscular lipids are presented in Table 1. There were considerable variations in the amounts of total fat in each of the three muscle locations examined. These variations were probably a reflection of factors such as breed, sex, weight and diet.

The neutral fat was the predominant fraction in all the samples, ranging from 68.61% in the semitendinosus to 95.53% in the longissimus dorsi, expressed as a percentage of total fat. Phospholipid material, ranging from 30.33% in the semitendinosus to 3.71% in the longissimus dorsi, accounted almost completely for the remaining total fat. A very small quantity of pigmented non-lipid material was obtained from all the samples (< 1% of total fat).

On examining the range of values obtained for the lipid fractions from each muscle, it can be seen from the correlation coefficients in Table 2, that the amount of neutral fat present in a given muscle was directly related to the

Table 1. Effect of location on contents of lipid fractions in 100 g of muscle tissue.[1]

Muscle	Total fat		Neutral fat		Phospholipid	
	Range (g)	Mean (g)	Range (g)	Mean (g)	Range (g)	Mean (g)
Semitendinosus	1.95– 6.24	3.48[a,b]	1.34– 5.64	3.02[a,b]	0.25–0.59	0.43
Longissimus dorsi	4.12–11.34	6.21[a]	3.68–10.84	5.62[a]	0.39–0.69	0.54
Triceps brachii	3.89– 9.97	6.48[b]	3.38– 9.46	5.75[b]	0.37–0.68	0.53

[1] Means within the same fraction with similar superscripts are significantly different ($P < .01$).

amount of total fat. However, the phospholipid fractions showed only slight variations from animal to animal within each muscle, and even these variations were not related to the total fat content of the muscle as evidenced by the low correlation coefficients reported in Table 2. As a result of this relatively constant amount of phospholipid material, the percentage contribution of the phospholipids to the total fat content decreased as the amount of total fat in the muscle increased. This effect was shown diagrammatically in a similar study by Turrki *et al.* (1967).

When the mean weights of the lipid fractions at the three different locations were compared, the semitendinosus muscle contained significantly smaller ($P < .05$) amounts of both total fat and neutral fat than either the longissimus dorsi or triceps brachii, in spite of the wide range in values obtained at each location. However, the phospholipids, in addition to showing little variation from animal to animal (Table 1) also did not differ significantly with regard to location, and a mean value of approximately 500 mg was reported for each muscle.

Turrki *et al.* (1967) reported similar values for the phospholipids in the extensor carpi radialis and psoas major muscles of beef. They did, however, find that there were small but statistically significant differences between the phospholipid contents of the two muscles. It was suggested that variations in the red and white fiber contents might account for these differences. Due to this result, and also the lower total fat content of the semitendinosus muscle, the mean percentage of phospholipids in the total fat of the semitendinosus muscle was significantly greater ($P < .05$) than the mean percentages found in either the longissimus dorsi or the triceps brachii (Table 3).

The two heads of the triceps brachii muscle were included in the samples removed from each carcass. On taking the samples out of storage for analysis, some seam or intermuscular fat could be observed between the lateral and medial heads. Since the actual fat within the muscle tissue appeared to be present in very small amounts, it is possible that without the seam fat the lipid fractions would have been similar in composition with those from the semitendinosus.

Callow *et al.* (1956) in their studies on the iodine numbers of the adipose tissue fat and intramuscular fat of beef animals also observed that the amount of phospholipid material present in muscle tissue was relatively constant at about 0.5 g per 100 g of muscle tissue. The iodine number increased with decreasing fat content in the case of both the intramuscular fat and the adipose tissue fat. While the relationship was linear with respect to the adipose tissue fat, it was hyperbolic in the case of the intramuscular fat. Callow *et al.* (1956) reasoned that two effects were responsible for the hyperbolic relationship. When the intramuscular fat content is low, the iodine value of the intramuscular fat asymptotically approaches that of the phospholipids, but when large amounts of fat are present in the muscle the iodine value of the intramuscular fat asymptotically approaches that of the adjoining subcutaneous fat.

The fatty acid composition of the lipid fractions

Qualitative identification. The fatty acid composition of the neutral fat and phospholipid fractions from the three muscles is shown in Table 4. Since standard fatty acid methyl esters were not available for C14:1, Cbr-16, C17:1 and C20:3, it was decided to analyze samples from three of the seven animals by gas chromatography using both the non-polar substrate Apiezon L and the polar substrate, diethylene glycol succinate. A mixture of the available standard fatty acid methyl esters was separated on the two different columns, and by plotting the $\log_{10}$ retention times relative to C16 obtained on the Apiezon L column against those obtained on the diethylene glycol succinate column, a grid was formed as described by James (1959). By referring to this grid, it was possible to tentatively identify the unknown fatty acid methyl esters.

There were no qualitative differences in the composition of the fatty acids in corresponding fractions at different locations. However, when at each location fatty acids

Table 2. Simple correlation coefficients between lipid fractions in beef muscle.

Location	Source of variation[1]	Total fat
Semitendinosus	Neutral fat	0.994**
	Phospholipid	0.013
Longissimus dorsi	Neutral fat	0.994**
	Phospholipid	0.004
Triceps brachii	Neutral fat	0.997**
	Phospholipid	0.035

[1] Wt. per 100 g muscle tissue.
** $P < .01$.

Table 3. Phospholipids and neutral fat fractions of beef muscle expressed as a percentage of total fat.[1]

Muscle	Neutral fat		Phospholipids	
	Range (%)	Mean (%)	Range (%)	Mean (%)
Semitendinosus	68.61–91.25	82.14[a]	7.90–30.33	14.36[a,b]
Longissimus dorsi	85.30–95.53	89.73	3.71–14.10	9.41[a]
Triceps brachii	84.49–94.86	90.16[a]	4.98–14.46	9.13[b]

[1] Means within the same fraction with similar superscripts are significantly different ($P < .05$).

Table 4. Effect of location on fatty acid composition of lipid fractions in beef muscle.[1]

Fatty acids[2]	Neutral fat			Phospholipids		
	Semi-tendinosus (%)	Longissimus dorsi (%)	Triceps brachii (%)	Semi-tendinosus (%)	Longissimus dorsi (%)	Triceps brachii (%)
C 10:0	0.12	0.13	0.12	.....	.....	.....
C 12:0	0.12	0.11	0.12	.....	.....	.....
C 14:0	3.48^{v}	4.24^{v}	3.60	0.28	0.36	0.24
C 14:1	1.01	1.29	1.18	.....	.....	.....
C 15:0	0.36	0.57	0.37	0.30^{v}	0.59^{vy}	0.32^{v}
Cbr-16:0	.....	.....	.....	0.16	0.16	0.15
C 16:0	28.07	28.31	27.12	18.16^{x}	22.55^{xv}	18.66^{v}
C 16:1	3.47	3.94	3.84	2.42	2.51	2.25
C 17:0	1.22	0.76	0.92	0.81^{v}	0.41^{v}	0.44
C 17:1	0.97	0.71	0.84	1.06	0.92	0.93
C 18:0	14.10	14.08	13.62	9.68^{y}	7.78^{yz}	9.97^{z}
C 18:1	44.03	43.25	45.67	23.11	24.34	25.38
C 18:2	2.05	1.78	1.75	23.85	23.02	22.59
C 18:3	0.94	0.96	0.80	1.44^{v}	2.00^{v}	1.61
C 20:3	.....	.....	.....	3.36	2.90	3.39
C 20:4	.....	.....	.....	15.23	12.54	14.21
Total saturated	47.47	48.20	45.87	29.39	31.85	29.78
Total monounsaturated	49.48	49.19	51.53	26.59	27.77	28.56
Total polyunsaturated	2.99	2.74	2.55	43.88	40.46	41.80
Total unsaturated	52.47	51.93	54.08	70.47	68.23	70.36

[1] Means on the same line with similar superscripts are significantly different ($P < .05$) for v and x, $P < .01$ for y and z).
[2] Calculated as % of total fatty acids (mean values from 7 animals).

identified in the neutral fat were compared to those identified in the phospholipids, some differences were noted. The fatty acids C20:3, C20:4 and a trace of Cbr-16:0 were present in the phospholipids but they had no counterparts in the neutral fat. Similarly C14:1 was present in the neutral fat fractions, but it was absent from the phospholipids.

Some of the minor fatty acids identified in this study do not correspond with those found by Hornstein *et al.* (1961). These workers used the polar column, polyvinylacetate, to analyze the fatty acid methyl esters of the lipid fractions from beef muscle. In the present study when the fatty acid methyl esters of both the phopholipids and neutral fats were separated on the polar substrate, diethylene glycol succinate, a peak appeared on the chromatogram with a relative retention time of 0.72 (retention time relative to that of C16:0). On consulting the grid it was found that this relative retention time could represent either pentadecanoic acid (C15:0) or tetradecadienoic acid (C14:2).

When the fatty acid methyl esters were run on the nonpolar column, Apiezon L, a peak appeared with a relative retention time of 0.68 which corresponded closely with that found on the grid for C15:0 but which was quite far removed from the grid value of 0.36 for C14:2. It is possible that there were slight traces of C14:2 present, as when brominated samples were separated on the diethylene glycol succinate column it was found that there was a small decrease in size in the peak assigned to C15:0 on each chromatogram. Hidaka *et al.* (1965) identified the fatty acid C15:0 during the course of their work on beef muscle lipids.

It can also be seen from Table 4 that C17:0 and C17:1 were present in both lipid fractions. Although they had not previously been identified in the work of Hornstein *et al.* (1961) their presence in beef intramuscular lipids was reported by Hidaka *et al.* (1965). Again, added evidence was obtained to identify C17:1 when the peak assigned to this acid disappeared when brominated samples were fractionated. Ziegler *et al.* (1967) reported the presence of both C17:0 and C17:1 in the lipids of ovine muscular tissue.

Quantitative Analysis. The results obtained for the fatty acid composition of the neutral fat and phospholipid fractions at each location were then compared from a quantitative viewpoint. As shown in Table 4, the phospholipids contained a much higher percentage of unsaturated fatty acids than the neutral fat fractions. This finding was in general agreement with the work reported by Hornstein *et al.* (1961).

There was only one significant effect due to location on the fatty acid composition of the neutral fat fractions. There was significantly more ($P < .05$) C14:0 in the longissimus dorsi muscles than in the semitendinosus, but the effect was not large enough to cause any significant difference between the total amounts of saturated fatty acids found in the two muscles.

In the phospholipid fractions the main effects associated

with location were an increase of C16:0 ($P < .05$) and a decrease of C18:0 ($P < .01$) in the longissimus dorsi, compared to either the semitendinosus or triceps brachii muscles. These two effects tended to balance one another, and they therefore had little effect on the overall degree of saturation at the different locations. Significant differences between locations were also found for the fatty acids C15:0, C17:0 and C18:3.

Hornstein *et al.* (1961) reported a value of 13.2% for the fatty acid C16:0 in the phospholipid fraction compared to values of 18.16–22.55% found in the present study. Since these workers reported a value of 15% for C18:0 compared to the values of 7.8–10.0% observed in the present work, there was no real difference between the two sets of data as regards the total content of saturated fatty acids. Similarly, the content of C20:4 found by Hornstein *et al.* (1961) was 5–7% higher than the amount observed in this work, but since higher percentages were reported for the other polyunsaturated fatty acids found in this study, the totals for polyunsaturated fatty acids were approximately the same in each instance.

Over-all, the longissimus dorsi intramuscular lipids appeared to be slightly more saturated than those of the triceps brachii or semitendinosus. This was true for both the phospholipid and neutral fat fractions. That the effect was not found to be significant may be due to the fact that the analyses of variance indicated a number of significant differences between animals in the fatty acid composition of the lipid fractions.

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Kinetics of the Enzymatic Development of Pyruvic Acid and Odor in Frozen Onions Treated with Cysteine C-S Lyase

SUMMARY—Extracts of commercially frozen onion, although possessing considerable peroxidase and catalase activity, were devoid of strong flavor and of L-cysteine sulfoxide lyase activity, the enzyme considered to be responsible for the development of onion flavor. These extracts, having been shown to retain the precursor(s) to such flavor, were selected as model *in situ* substrates for a study of the kinetics of odor production catalyzed by the L-cysteine C-S lyase of *Albizzia lophanta* seed endosperm. The results suggest that both odor and pyruvic acid may be produced via the same enzyme but that the odor is formed after the formation of pyruvic acid. From the data it has been calculated that the odor threshold value of some of the enzymatically produced odor-bearing constituents in onions may be less than one part per billion.

INTRODUCTION

THERE EXISTS A WEALTH of evidence that the flavor of many foods arises as a consequence of the interaction of enzyme and substrate (flavor precursor) when the cellular integrity of the food tissues is destroyed as the result of comminution or bruising (Schwimmer, 1963a).

In the case of onions, there is a very close correlation between the content of pyruvic acid, one of the products of the relevant onion enzyme reaction, and the odor intensity of freshly comminuted and of reconstituted dehydrated onions (Schwimmer *et al.*, 1961; Schwimmer *et al.*, 1962; Schwimmer *et al.*, 1964a).

The kinetics of this type of enzyme action (L-cysteine [sulfoxide] C-S lyase), as measured by rate and extent of pyruvic acid production has been studied in detail with enzyme preparations from onion (Schwimmer *et al.*, 1963; Schwimmer, 1963b, 1964; Schwimmer *et al.*, 1964b) and from the seeds of *Albizzia lophanta* (Schwimmer *et al.*, 1960). However, no study has been published of the kinetics of enzymatically induced odor formation, as measured by olfactory thresholds. Such a study should be of value in further probing the validity of our understanding of the relationship between odor intensity and enzyme action.

The present report constitutes an investigation of the course and rate of odor production as affected by the variables of time, enzyme concentration, and substrate concentration. The same enzyme reaction mixtures were used for traditional kinetic studies of the enzymatic production of pyruvic acid, using commercially frozen onion as source of substrate and a preparation of the L-cysteine sulfoxide C-S lyase of the endosperm of *Albizzia lophanta* seeds as source of enzyme.

MATERIALS AND METHODS

WE PREPARED THE ENZYME from seeds of *Albizzia lophanta* (Schwimmer *et al.*, 1960). This enzyme acts on S-substituted derivatives of both L-cysteine sulfoxides in contrast to the onion enzyme whose range of specificity is limited to L-cysteine sulfoxide derivatives. The seed (170 g) was ground in a Wiley mill, and mixed the resulting meal with 2 liters of trichloroethylene in a 2-L graduated cylinder. The yellow enzyme-containing endosperm meal was then removed, devoid of seed coat, which collected at the top, air dried, and extracted at 5°C with 1 L of 80% ethanol to remove the bulk of the endogenous substrate (djenkolic acid). After the suspension was filtered, the filter cake was washed successively with 95% absolute ethanol and dried in a desiccator. Then the dried preparation was stirred with 350 ml H_2O at 5°C for 3 hr and squeezed through 4 layers of cheesecloth. The extract was centrifuged at 0°C for 20 min at 16,300 × G and the resulting supernatant liquid was recentrifuged at 31,000 × G for 45 min. Ninety ml of absolute ethanol was added to the final supernatant liquid and the precipitate was centrifuged off and allowed to dry in a vacuum desiccator to yield 4.5 g of enzyme preparation.

S-propyl-L-cysteine sulfoxide was a synthetic mixture of the (+) and (−) diastereomers (Schwimmer *et al.*, 1964b).

For most of the experiments, onion extracts were prepared by blending 50 g of commercial frozen onions with 50 ml H_2O and squeezing the resulting homogenate through 4 layers of cheesecloth. Thus, each ml of extract represented about 500 mg of frozen onions. Heated onion extracts were prepared by adding 50 g of frozen onion to an equal volume of boiling water, heating at 100°C for 5 min, adjusting the volume to 100 ml and squeezing through cheesecloth. For the detection of catalase and peroxidase, 2 ml of onion extract were added to 6 ml of 0.5% H_2O_2 (catalase) or 0.5% H_2O_2 + 0.167% guaicol (peroxidase) (Woodruff, 1947).

For estimation of enzymatic production of pyruvic acid and odor, 2 ml of enzyme reaction mixture containing onion extract and enzyme solution were incubated at 25°C. Each ml of reaction mixture (incubated 1 hr at 25°C) for Table 2 contained, when added: onion (or heated onion) 200 mg; substrate (S-propyl-L-cysteine sulfoxide), 5 μmoles; enzyme, 1 mg; buffer (TRIS, pH 8.5), 0.1 mmole. For the experiments shown in Fig. 1, each ml of reaction mixture contained 250 mg of onion where enzyme was varied, and 1.25 mg of enzyme where time was varied. For Fig. 2, the onion concentration was 250 mg/ml. For Figs 3 and 4, the concentrations of enzyme were 2.5 and 1.25 mg/ml respectively.

The C-S lyase enzyme reaction was terminated by addition of 0.5 ml of 1 *N* HCl and, after 30 min, 7.5 ml of

H_2O was added. One-half ml aliquots of the acid-treated dilution reaction mixtures were used for the determination of pyruvic acid. Separate control incubation mixtures contained either no enzyme or no onion extract. The remainder of the acid-treated reaction mixture which had been diluted to 10 ml was used for estimation of odor thresholds as described in detail in Schwimmer *et al.* (1962).

The odor in one ml of reaction mixture diluted to the threshold value is arbitrarily defined as one odor unit. Total odor units in 2 ml of the original enzyme incubation mixture are therefore equal to the total volume (in ml) after dilution to the odor threshold. Thus, if the 10 ml of acid-treated reaction mixture (10 ml from 2 ml of original enzyme reaction mixture) has to be diluted 1,000-fold to achieve the odor threshold concentration, total odor units = 10,000. Odor units per ml of original enzyme reaction mixture equals one-half this value.

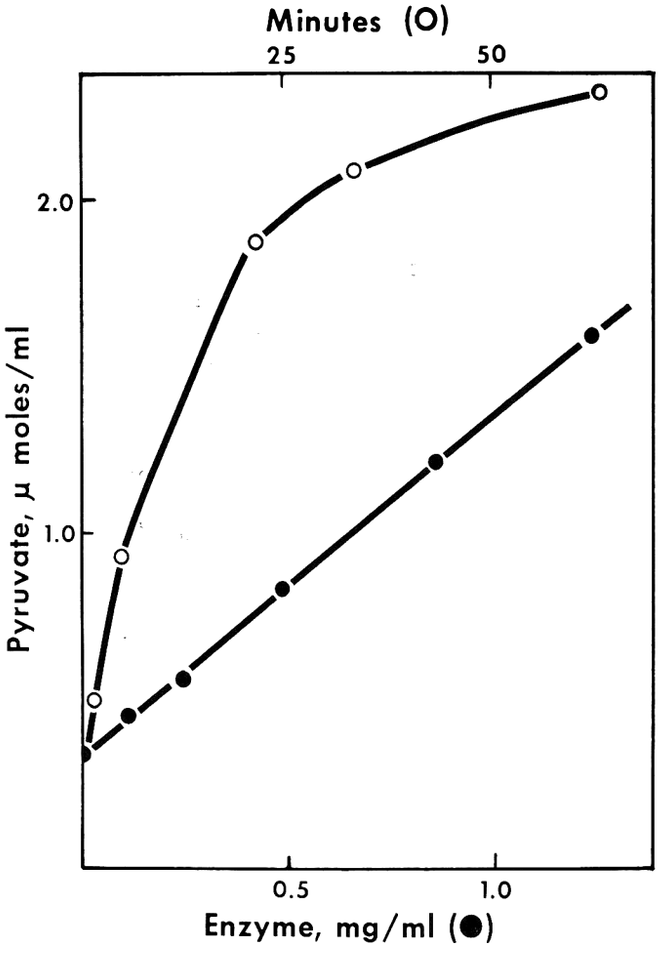

Fig. 1. Pyruvic acid content of extract of frozen onion treated with enzyme as function of time and enzyme concentration.

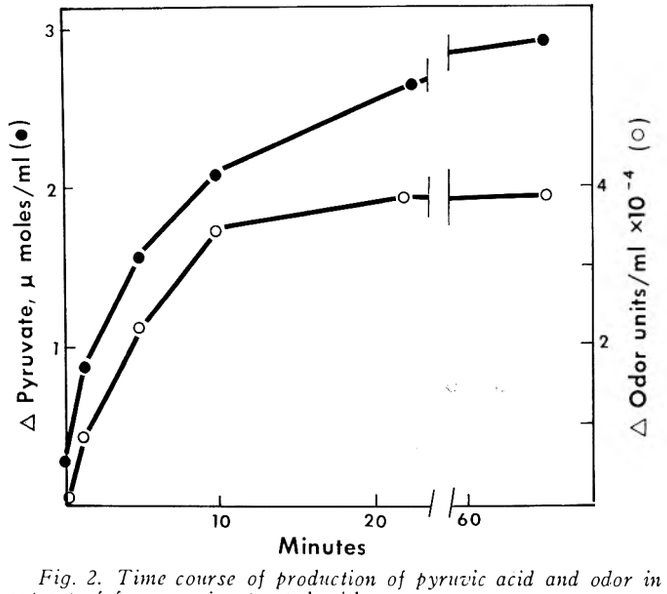

Fig. 2. Time course of production of pyruvic acid and odor in extract of frozen onion treated with enzyme.

RESULTS

THE THAWED ONIONS, although not as turgid as freshly diced onions, were considerably firmer than diced raw

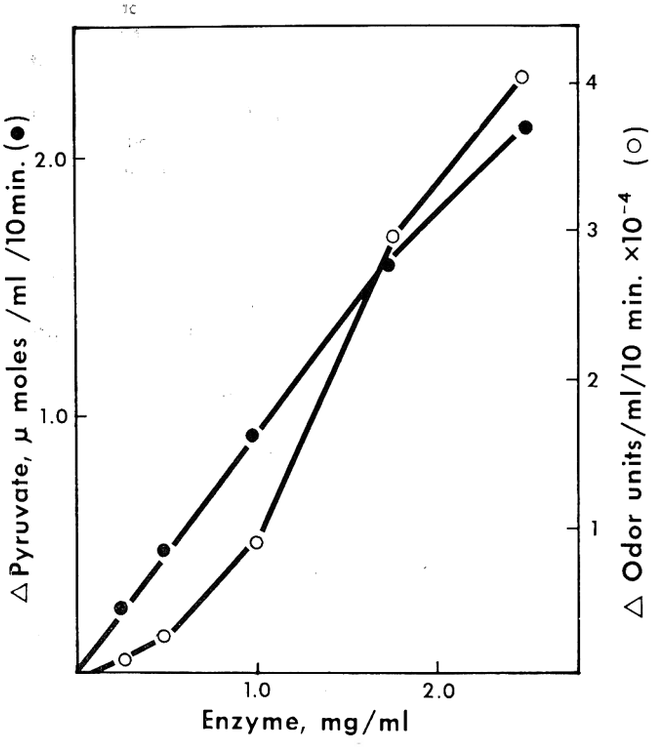

Fig. 3. Rate of formation of pyruvic acid and odor as a function of enzyme concentration.

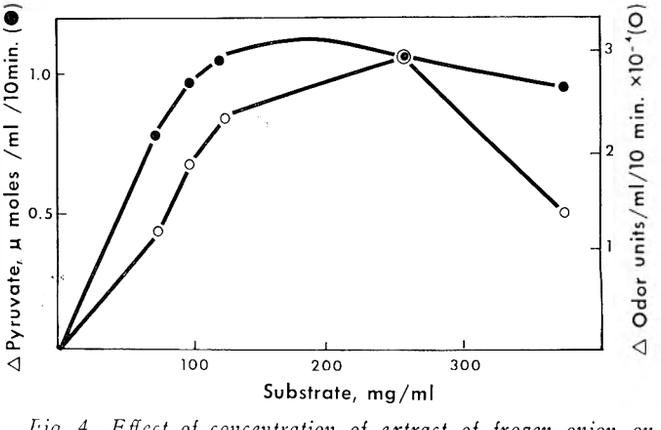

Fig. 4. Effect of concentration of extract of frozen onion on rate of formation of pyruvic acid and of odor.

Table 1. Test for presence of catalase and peroxidase in extracts of frozen onion.

Treatment or addition			Observation	
H_2O_2	Guaiacol	Heat	O_2 evolution (catalase)	Coloration (peroxidase)
−	−	−	−	−
+	−	−	+	−
+	−	+	−	−
+	+	−	−	+
+	+	+	−	−
−	+	−	−	−

onions subjected to a 5-min heat treatment by steam or radio frequency radiation. Furthermore, extracts from the frozen onion gave strong positive tests for peroxidase and catalase (Table 1), thus suggesting that the onions were not blanched prior to freezing. On the other hand, although having a pleasant mild odor and taste (*cf.* Woodruff *et al.,* 1947), mastication or comminution of these frozen onions did not develop the intense sensory attributes of raw onion macerates, i.e.: lachrymator effect, astringency, highly pungent odor, and bitterness, and subsequent pink coloration. After thawing, unblanched frozen onions were intensely bitter (Schwimmer, 1967). This would suggest that the precursors, the enzyme, or both of these moieties of the enzyme system responsible for the development of the typical onion flavor were missing. To test for these possibilities, onion extracts were treated with a typical onion precursor (S-propyl-L-cysteine sulfoxide), enzyme (C-S lyase of *Albizzia lophanta*) and/or a buffer which changed the extract to a more favorable pH for C-S lyase action (Schwimmer *et al.,* 1963).

Changes were measured by the development of pyruvic acid and by olfactory threshold tests. The results (Table 2) demonstrate that the frozen onions had a very low, almost vanishing, enzyme content demonstrable only by shifting to a more favorable pH. They did, however, contain considerable precursor to both pyruvic acid and odoriferous volatiles. The pyruvic acid values for the controls containing onion only were about the same as those for fresh onions heated to prevent enzyme action (Schwimmer *et al.,* 1962). This indicates that little or no interaction of enzyme and substrate occurred during processing and freezing of the onions. The values obtained in the presence of enzyme are in the range of those for a moderately strong onion (Schwimmer *et al.,* 1962).

Table 2. Demonstration that odor precursor but not enzyme is present in commercially frozen onion.

System	Pyruvic acid μmole/g onion		Threshold ppm (pH 5.8)
	pH 5.8	pH 8.0	
Onion	1.6	1.8 (2.5)[1]	230
Heated onion	1.6	.....	.....
Onion + substrate	1.6	2.0	250
Onion + enzyme	8.2	8.3	9
Heated onion + enzyme	8.7	.....	.....
Enzyme + substrate	.....	30.8	.....
Enzyme + onion + substrate	.....	22.8	.....

[1] Frozen onion blended with TRIS buffer.

Fig 1 shows the effect of enzyme concentration and of time on the pyruvic acid content of an unbuffered enzyme reaction mixture containing onion extract and enzyme.

Figs. 2, 3 and 4 show the effects of varying time, enzyme concentration, and substrate (onion) concentration, respectively, on the development of pyruvic acid and odor. The data on odor are not precise enough to permit a quantitative interpretation of the enzyme kinetics. However, inspection of the data suggests qualitative consistencies in the relation between pyruvate and odor. Both pyruvate and odor development increased with increasing values of these variables, except at very high substrate concentrations (Fig. 4).

The data on the effect of substrate concentration (Fig. 4) suggest that the substrate concentration at half maximum rate (i.e. K_m) of both odor and pyruvate production are of the same order of magnitude (*ca* 100 mg of onion per ml of reaction mixture). At low values of the three variables, the odor production appears to lag behind the production of pyruvate. Although the observed lag for any one variable would not in itself be significant, the fact that it appears with all three variables indicates that it may be real.

In view of previous knowledge of the enzymatic mechanism of odor production from S-substituted-L-cysteine sulfoxides (Virtanen, 1965; Schwimmer, 1967a; Carson, 1967) the data presented support the interpretation that both odor and pyruvate are produced via the same enzyme reaction. The apparent lag of odor development in comparison with pyruvate production at low values of time, enzyme and substrate concentrations suggests that pyruvic acid is formed before the odoriferous products. This is consistent with the current formulation of the reactions leading to such products. Thus the primary products are considered to be pyruvic acid, ammonia, and the methyl, propyl and propenyl derivatives of sulfenic acid.

It is the latter unstable moieties which, via a series of condensations and dismutations and eliminations, are believed to give rise to the odor-bearing volatiles characterizing onion odor. Although the temporal separation of these two classes of reaction may actually be quite small, they could be magnified during the interval between the time of addition of acid to the reaction mixture and the organoleptic evaluation. During this time the enzyme is no longer producing pyruvate but non-enzymatic reactions leading to more odor could occur. These reactions would be slower at low levels of reactants.

The data presented here afford a theoretical means of calculating the odor threshold concentrations of the odoriferous substances. It is assumed that only the RSO radicals of L-cysteine sulfoxide derivatives are converted to odoriferous substances. From the data presented here, one can estimate that there are about 10 μmoles of substrate per g of onion. Since the smallest odor threshold value found was 5 ppm (5 μg of onion per ml) the average concentration of odoriferous substances at the threshold was about 2 to 4 nanograms per ml or 3 parts per billion. Since we are dealing with a mixture of substances of varying odor intensity, the threshold of the most odoriferous substances from onion may actually fall in the range below one part per billion.

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The Free Amino Acids of Israel Orange Juice

SUMMARY—Twenty-two Israel orange juice samples were analyzed chromatographically, and 16 free amino acids were identified with seven different solvent systems. Aspartic acid, glutamic acid, lysine, alanine, and proline were identified with all 7 solvent systems; asparagine with 6; serine with 5; arginine, valine and leucine with 4; γ-amino-butyric acid with 3; glycine, methionine and phenylalanine with 2; and threonine and tyrosine with 1. The presence of isoleucine in Israel orange juice appears doubtful.

A quantitative estimation of the free amino acids indicates that amounts of aspartic acid, serine, and alanine are high compared with California orange juice, but glutamic acid and lysine are low.

INTRODUCTION

A KNOWLEDGE of the amino acid content of citrus juices may assist industry not only in estimating the quality of their products and the maturity of the initial fruit but also help in study of the darkening of the products. Safina (1964) suggested application of chromatographic separation of amino acids as an indicator of the quality of citrus juices or beverages. Rockland *et al.* (1950) postulated the importance of the reaction of amino acids with sugars in the browning of processed citrus products. A number of workers (Rockland *et al.*, 1954; Wedding *et al.*, 1955; Rockland *et al.*, 1955) have investigated the use of amino acids as a possible index of maturity. Although the amino acids of orange juice generally do not belong to the essential ones, Rockland (1961) suggests that the citrus amino acids may contribute to some extent to the nutritional value of citrus juices.

The amino acid content of the California Valencia orange has been studied in detail (Rockland, 1961), while knowledge is also available for the Washington Navel orange (Rockland, 1961) and in less detail for two Japanese varieties (Ito *et al.*, 1952), for Italian oranges (Safina, 1953) and for Italian blood oranges (Wucherpfennig, 1966). Work of Wedding *et al.* (1954) has indicated both qualitative and quantitative differences between Valencia and Washington Navel oranges, particularly with regard to the absence of γ-amino butyric acid and the relatively large quantity of tyrosine in the juice of Washington Navel oranges.

No knowledge is yet available on the amino acid content of the Israeli Shamouti orange and the extent of variations due to its maturity, season and location. This study was initiated as part of an investigation into the detection of adulteration of citrus juices and citrus juice products.

EXPERIMENTAL

Preparation of samples

Shamouti and Valencia orange fruit were collected from six groves of different locality and age at different maturity dates (Table 1). Fifty fruits were selected at random from three trees in the center of the grove. The fruit was transported in open containers and processed within 24 hr of harvest. Physical measurements were made on the fruit as shown in Table 1, and its juice extracted with a stainless-steel electrical hand reamer and strained through a 16-mesh stainless-steel wire screen. The orange juice was pasteurized at 95°C for 50 sec in a rotating falling-

Table 1. Description of groves of Shamouti oranges and composition of their fruits.

Rootstock	Grove and location	Age of tree (years)	Harvest date	pH	Acidity as citric acid %	Brix %	Formol value ml. N.NaOH per liter juice
(1) Sour orange	Gesher Haziv	35	Nov. 18	3.0	1.57	10.7	10.0
	West Gallilee		Nov. 29	3.0	1.66	10.4	9.0
			Jan. 2	3.3	1.22	11.0	14.0
(2) Sour orange	Rassco Chamama	6	Dec. 5	3.0	1.84	10.5	12.5
	Ashkelon		Jan. 9	3.0	1.72	10.9	11.0
			Jan. 23	3.0	1.67	11.3	13.5
(3) Sweet lime	Galia, Rehovot	35	Dec. 20	3.1	1.08	9.8	18.0
			Jan. 12	3.4	1.88	9.8	25.0
			Jan. 27	3.4	0.91	10.1	17.0
(4) Sour orange	Ein Harod	30	Nov. 13	3.2	1.28	11.0	10.0
	Beit Shean		Jan. 2	3.4	1.12	11.2	18.0
(5) Sour orange	Cabri	30	Dec. 3	2.9	1.86	10.8	17.0
	N.W. Galilee		Dec. 17	3.2	1.63	10.7	16.0
(6) Sour orange	Galia, Rehovot	35	Dec. 20	3.0	1.23	10.3	17.0
			Jan. 27	3.3	1.09	9.8	18.0

film steam-heated pasteurizer and filled hot into heat-sterilized, hot, dark-colored bottles. The sealed bottles were inverted to sterilize the seals, cooled, and stored for 1 month at room temperature. Rockland *et al.* (1956) have shown that, within the normal range of pasteurization times and temperatures followed by storage for 5 months at 100°F, no significant changes in the levels of 7 free amino acids were detectable.

The stored processed juice samples were filtered by suction through a Buchner funnel on Whatman No. 1 filter paper. Filter-aid-treated juices gave identical results to untreated juices, but use of the latter was more convenient in the laboratory. The purification procedure of Safina *et al.* (1959), however, did not give all the amino acids that the filtered juice gave and was not employed in the analysis.

Chemical analysis

The pH of each juice was measured electrometrically with a Beckman glass-electrode pH meter standardized with pH 7.00 buffer. Acidity was determined by titration of 10 ml of the filtered juice with 0.01*N* sodium hydroxide with an alcoholic solution of phenolphthalein used as indicator and the acidity expressed as citric acid. Brix measurements were made with a Zeiss Abbé refractometer, corrections being made for temperature. The formol value was estimated by the method of Safina (1964).

Filter paper chromatography

All juices were analyzed by filter paper chromatography. Preliminary experiments were carried out on two of the juices to determine the most suitable working conditions. Paper chromatography was carried out by a one-dimensional ascending technique on Whatman No. 1 filter paper sheets of size 57½ × 56½ cm always along the machine direction. Three solvent systems were used: 1) Butanol-acetic acid-water (4:1:5 v/v); 2) phenol-sodium citrate-KH_2PO_4 (6.3% sodium citrate and 3.7% KH_2PO_4 per 100 g phenol); 3) propanol-water (70:30 v/v).

Additional solvents were used on two juice samples only to confirm the presence of certain amino acids. These solvents were: 4) *N*-butanol-acetic acid-water (4:1:1 v/v), which is specific for phenylalanine (Rauen, 1956); 5) phenol-*n*-propanol-water (100:20:20 v/v), which was used to identify threonine; 6) methylethyl ketone-propionic acid-water-tert. butanol (75:25:30:20 v/v), specific for methionine and glutamine (Furuholmen *et al.*, 1964; Lederer *et al.*, 1955); 7) *N*-butanol-ethanol-water (4:1:1), which was used to identify and confirm methionine and phenylalanine.

In addition, two-dimensional chromatography was carried out for confirmation, using first solvent No. 2 and then solvent No. 1.

The ascending one-dimensional chromatographic technique consisted of spotting 6 ml of the filtered juices 5 cm from the bottom of the filter paper and drying each spot in a stream of warm air. Juices were spotted in triplicate on the same paper 5 cm from each other and adjacent to spots of pure compounds both individually and as a mixture. Three papers were run for each juice in each solvent. The atmosphere of the developing glass chamber was allowed to equilibrate with the solvent 12 hr prior to development, and fresh solvent was used for each run. The papers were run for 24 hr, removed, and allowed to dry at room temperature (approx. 30°C), and then run again for an additional 24 hr in the case of solvents Nos. 2, 4 and 5, and with the 2-dimensional run, while for solvents No. 1, 3 and 6, papers were run for an additional 24 hr (i.e. 72 hr in all).

Color development was attained by an alcoholic solution of ninhydrin applied as an aerosol spray. R_f values were calculated according to the final solvent front.

Identification of the amino acids was made according to: 1) R_f values compared with those of pure compounds run under the same condition; 2) ninhydrin color reactions (spot colors)—compared with those of the pure compounds and to literature; and 3) comparison of results of the three solvent systems and the additional specific solvent systems used.

Pure samples of serine, proline and arginine were not available and identification was made by comparison with R_f values from literature and by ninhydrin color reactions.

Table 2. The free amino acids identified in Israel orange juice. R100F values.

	One-dimensional paper chromatography							2-Dimensional paper chromatography*	One-dimensional thin layer chromatography*
Solvent used	1	2	3	4	5	6	7	1, 2	1
Aspartic Acid	34(34)	24(18)	49(50)	24(30)	10(11)	10(11)	4(4)	+	+
Glutamic Acid	46(44)	48(43)	54(54)	32(37)	17(18)	25(26)	2(3)	+	+
Serine***	40 or glycine	55	62	46	29	—	12	+	+
Asparagine	26(23)	64(65)	51(52)	18(20) or arginine	35(37)	15(16)	6(6)	+	+
Arginine***	23	—	45	18 or asparagine	—	4	5	—	+
Lysine	15(17)	76(76)	43(46)	11(12)	75(76)	2(4)	1	+	+
Alanine	51(51)	85(84)	71(71)	41(45)	—	35(37)	+	+	+
Y-Amino Butyric	65(64)	94(93)	—	54(55)	—	—	—	+	+
Proline***	56	99	76	47	90	43	26	+	+
Glycine	40(38) or serine	—	64(63)	—	—	12(13)	—	+	+
Valine	75(74) or methionine	—	85(86)	67(66)	—	49(50)	36(36)	+	+
Leucine	84(86) or isoleucine	—	88(87) or isoleucine	80(80)	86(87)	83(85)	62-63(61)	+	+
Methionine	75(74) or valine	—	—	—	—	60(61)	45(43)	+	+
Phenylalanine	—	—	—	73(72)	—	74(71)	53(50)	—	+
Threonine	—	—	—	—	46(44)	—	—	—	+
Tyrosine	—	—	—	50**(49)	—	—	—	—	+ or isoleucine

* Indicating presence or absence of amino acids.
** Traces.
*** Pure samples of these amino acids were not available.

Thin-layer chromatography

Thin-layer chromatography was carried out on two juice samples in order to confirm the presence of phenylalanine, isoleucine, and tyrosine. The absorbent used was silica gel on glass plates and the solvent butanol-acetic acid-water (4:1:5) with a one-dimensional ascending technique for 2 hr twice after drying each time.

Quantitative analysis

A fully quantitative determination was carried out on six juice samples for eight of the free amino acids, using two solvent systems No. 1 and 3. Chromatographic runs were carried out as previously described, and the ninhydrin color spots were cut out from the papers and extracted in test tubes in 96% ethyl alcohol. The alcoholic extracts were then measured colorimetrically in a Klett and Summerson Colorimeter using Filter No. 55 (wave length 520–600 mμ). Quantitative estimations were then made by comparison of the results with standard curves previously obtained on pure compounds of the amino acids studied.

RESULTS AND DISCUSSION

THE FREE AMINO ACIDS identified in Israel orange juice are listed in Table 2. In all, 16 free amino acids were identified in the juices studied. Only aspartic acid, glutamic acid, alanine, lysine and possibly proline were identified and confirmed in one or more solvent systems. The presence of isoleucine in Israel orange juice appears doubtful. Solvents No. 1 and 3 with one-dimensional paper chromatography indicated the presence of either isoleucine or leucine, while leucine was identified in solvents Nos. 4, 5, 6, and 7. Again, tentative identification of isoleucine or tyrosine was made with thin-layer chromatography. However, since tyrosine was identified also with solvent No. 4, it seems that isoleucine does not occur in Israel orange juice.

The R_f values of the identified amino acids according to solvent systems No. 1, 2, and 3 represent averages from analysis of the 22 different orange juice samples. The R_f values listed for solvent systems No. 4, 5, 6 and 7 were obtained from only two of these samples, analyzed primarily for confirmatory evidence. The values given for the thin-layer chromatographic analysis were those from one juice sample only, which was employed specifically for the confirmation of phenylalanine and secondarily for tyrosine. The values in parentheses are those obtained with the pure compound, where available.

It will be seen that the reproducibility of the R_f values differs somewhat with each of the three principal solvent systems used. Solvent systems No. 1 and 2 show high

Table 3. Quantitative estimation of some free amino acids in Israel processed orange juice.

	Concentration (mg/100 ml. juice)					
	Israel Shamouti[1]		California Valencia[2]		California Navel[2]	
Amino acid	Range	Average	Range	Average	Range	Average
Alanine	34-38	36	13-25	17	18	18
Arginine	44-46	45	90-150	120	73-82	77
Aspartic Acid	105-125	115	32-94	71	41-44	42
Glutamic Acid	27-29	28	19-71	49	19-21	20
Leucine	5-7	6	(traces[3])	—	—	—
Lysine	7-13	10	(22[3])	—	—	—
Serine	67-73	70	4-36	15	23-30	26
Valine	11-13	12	(traces[3])	—	—	—

[1] Values from 6 samples of Israel Shamouti oranges representing 6 different groves selected at mid-season.
[2] Rockland (1959).
[3] Rockland (1961).

reproducibility, although with solvent system No. 2 the R_f values of the pure compounds of aspartic acid and glutamic acids are markedly lower than those of the juice. Solvent system No. 3 also shows fairly good reproducibility, and the R_f values of the pure compounds (except lysine) are in close agreement with those of the samples.

Such reproducibility of R_f values, with all of the solvent systems, can be considered as a reliable criterion of identity.

Table 3 records the concentration of eight amino acids in six samples as estimated by the colorimetric technique, with solvent systems No. 1 and 3. A comparison of these results with those obtained with California Valencia oranges and California Washington Navel (Rockland, 1959; 1961), shows that Israel Shamouti orange juice contains more alanine, more valine, more than twice the amount of serine, and slightly more leucine; but less arginine, glutamic acid, and lysine, than Valencia orange juice.

The Israel Shamouti orange juice also contains more alanine, serine and aspartic acid but less arginine than Calfornia Washington Navel orange.

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Rigor State, Freeze Condition, pH, and Incubation Temperature and Their Influence on Color Development and Extract Release Volume in Ovine Muscle Homogenates

SUMMARY—The effect of pH and incubation temperature on the development of filtrate color, and a modified extract release volume (ERV) were studied in unfrozen and frozen, pre- and post-rigor ovine muscle homogenates.

Buffer-pH, incubation temperature and the interaction between these two factors had a highly significant effect ($P < 0.01$) on filtrate color and ERV. Rigor state and the interactions, rigor state × buffer-pH, freeze state × buffer-pH, and freeze state × incubation temperature had a highly significant effect ($P < 0.01$) upon ERV after 24 hr incubation.

Pale colored filtrates developed in homogenates that were buffered at pH 5.2 and 5.6 and incubated at 20, 30, and 40°C. There was a significant correlation between ultimate pH and color scores for the filtrates obtained from homogenates incubated at 10, 20, 30, and 40°C but there was no relationship between the two variables when the homogenates were incubated at 0 and 5°C. The ERV decreased significantly ($P < 0.01$) with increasing incubation temperatures and pH of buffer. Pre-rigor homogenates at low pH levels released significantly smaller ($P < 0.01$) amounts of extract than did post-rigor tissue. Incubation temperature influenced the magnitude of the correlation between ultimate pH and ERV.

INTRODUCTION

NUMEROUS INVESTIGATORS, (Wismer-Pedersen *et al.*, 1961a,b; Bendall *et al.*, 1962; and Kastenschmidt *et al.*, 1964) have demonstrated that the development of pale, soft, exudative (PSE) porcine musculature is associated with the rapid decline of tissue pH at elevated temperatures. McLaughlin *et al.* (1963) reported that pre-rigor extracts of porcine sarcoplasmic proteins, buffered at various pH levels, became turbid and subsequently developed a pale color when incubated at elevated temperatures. They postulated that myoglobin became bound to the sarcoplasmic proteins and was subsequently precipitated.

Conflicting evidence is presented by Henry *et al.* (1955) and Lawrie (1960) who reported the absence of myoglobin in PSE tissue, while Briskey *et al.* (1959) were unable to demonstrate differences in myoglobin content in PSE and dark, firm and dry (DFD) tissue. Wismer-Pedersen (1959) postulated that variation in muscle color was independent of pigment concentration, and was due to differences in the spectral properties of light reflected from the cut surface of the muscle.

The limited amount of information that is available on factors associated with variations in color, water binding capacity and ERV of ovine and bovine tissue strongly implicates the influence of pH (Hall *et al.*, 1944; Hedrick *et al.*, 1959, 1961; Urbin *et al.*, 1961; Munns *et al.*, 1965).

The existence of a high inverse relationship between pH and water holding capacity, drip release, and ERV, in frozen and unfrozen post-rigor bovine tissue has been reported by Empey (1933), Sair *et al.* (1938), Bouton *et al.* (1957), Hamm (1963), and Jay (1964).

Similarly, it has been reported that the post-mortem interval prior to freezing (Sair *et al.*, 1938; Ramsbottom *et al.*, 1940) and the rigor phase of the tissue (Hamm, 1963) influence the water holding capacity.

This article reports a study on the effects of pH, incubation temperature, rigor state, freeze state and their interactions upon filtrate color and modified ERV of ovine muscle homogenates.

EXPERIMENTAL

Materials

Five cross-bred lambs—approximately 9 months old, of unknown genetic and nutritional history—were slaughtered after 12 hr starvation. The complete right longissimus dorsi muscle was removed from each animal within 10 min of slaughter (pre-rigor). The carcasses were held at 5°C, and 48 hr post-mortem the complete left longissimus dorsi was removed (post-rigor). After removal of all external connective tissue and fat the tissue was ground through a fine plate.

Methods

A 30-g sample of each muscle was mixed with 120 ml of chilled (3°C) 0.12 *M* cacodylic acid-sodium cacodylate buffer and homogenized at high speed for 2 min in a Servall Omni-mix. Buffered homogenates were prepared at 5 pH levels: 5.2, 5.6, 6.0, 6.4, and 6.8.

An aliquot of 60 ml from each buffered homogenate was transferred immediately into a plastic bag, frozen for 45 min in a dry ice-ethanol mixture and subsequently stored at −10°C for 24 hr. The remaining portion of buffered homogenates was prepared for immediate incubation at the different temperatures. Approximately one-sixth of each homogenate (frozen and unfrozen) was incubated in a ½-in. test tube for 24 hr at one of the following temperatures: 0, 5, 10, 20, 30, and 40°C. Phase separation during incubation was prevented by periodic shaking of all samples.

The procedure of Jay (1964) was modified for the determination of extract release volume. After 24 hr incubation, the homogenates were filtered at 5°C for 1 hr and

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Table 1. Mean pre- and post-incubation pH values for pre- and post-rigor muscle homogenates.[1]

	Buffer pH[2]														
	5.2			5.6			6.0			6.4			6.8		
Treatment	Pre	Post	Change	Pre	Post	Change	Pre	Post	Change	Pre	Post	Change	Pre	Post	Change
Pre-rigor	5.57[a]	5.23[a]	−0.34	5.78[a]	5.52[a]	−0.26	6.10[a]	6.07[a]	−0.03	6.42[a]	6.67[a]	+0.25	6.70[a]	6.90[a]	+0.20
Post-rigor	5.32[b]	5.17[a]	−0.15	5.65[b]	5.55[a]	−0.10	5.97[b]	6.47[b]	+0.50	6.31[b]	6.98[b]	+0.67	6.61[b]	7.11[b]	+0.50

[1] n = 60.
[2] Means of pre- and post-rigor homogenates within a buffer pH followed by same postscript not significantly different ($P < 0.05$).

the ERV was calculated by relating the weight of the filtrate to the weight of the homogenate filtered. Homogenate color was subjectively evaluated on the filtrate using a 10 point rating scale. A pale color (O.D. 540 mμ = 0.07) was rated as 1 and a dark red color (O.D. 540 mμ = 0.82) as 10. Pre- and post-incubation pH values were measured at each incubation temperature.

The experiment had a 2 × 2 × 5 × 6 × 5 factorial design and a standard analysis of variance was used. The sources of variation were tested for significance against the appropriate expected error mean square (Steel *et al.*, 1960). The percent ERV data were not transformed prior to analysis because their distribution was normal.

Table 2. Analysis of variance of pre-incubation pH values.

Source of variation	Degrees of freedom	Mean squares
Rigor state	1	3.16**
Buffer pH	4	28.21***
Animals	4	0.31**
Animals × rigor (Error 1)	4	0.27**
Animals × buffer pH	16	0.04
Rigor × buffer pH	4	0.13**
Animals × rigor × buffer pH (Error 2)	16	0.02
Total	49	

** $P < 0.01$.
*** $P < 0.001$.

Table 3. Mean post-incubation pH values for pre- and post-rigor frozen and unfrozen homogenates.[1]

	Freeze state[2]		
Rigor state	Unfrozen	Frozen	Mean and standard deviation
Pre-rigor	6.07^{a}X	6.01^{b}X	6.04 ± 0.36
Post-rigor	5.97^{a}Y	5.95^{b}Y	5.96 ± 0.41
Mean and standard deviation	6.01 ± 0.39	5.98 ± 0.39	

[1] n = 150.
[2] Means within a rigor treatment followed by same lower case superscript and those within a freeze state followed by same upper case subscript not significantly different ($P < 0.05$).

RESULTS AND DISCUSSION

Incubation of buffers

Cacodylic acid-sodium cacodylate buffers were used in this experiment because of their wide range (pH 5.0–7.4) and stable pKa over a wide range of temperatures (Dawson *et al.*, 1959). When the buffers were tested under the conditions used in this experiment, the post-incubation pH values were similar in all cases to those recorded prior to incubation.

Pre- and post-incubation homogenate pH

Table 1 presents the pre- and post-incubation pH values pooled within each buffer-pH treatment for pre- and post-rigor tissue. At all buffer-pH levels the pre-incubation pH values of post-rigor tissue were significantly lower ($P < 0.05$) than the corresponding pre-rigor sample. Pre-incubation pH values differed significantly ($P < 0.001$) among buffer groups and between rigor states ($P < 0.01$) and there was a highly significant ($P < 0.01$) interaction between them (Table 2).

Table 3 presents the mean post-incubation pH values for frozen and unfrozen, pre- and post-rigor homogenates. Table 4 presents means at each buffer-pH level and incubation temperature. Table 5 presents the analysis of variance. Rigor state ($P < 0.01$), buffer-pH ($P < 0.001$), freeze state ($P < 0.01$) and incubation temperature ($P <$

Table 4. Mean post-incubation pH value at each buffer pH and incubation temperature.[1]

	Incubation temperature (°C)[2]						
Buffer pH	0	5	10	20	30	40	Mean and standard deviation
5.2	5.49^{ab}	5.50^{ab}	5.49^{ab}	5.49^{ab}	5.52^{a}	5.46^{b}	5.49 ± 0.49
5.6	5.71^{a}	5.71^{a}	5.71^{a}	5.72^{a}	5.71^{a}	5.65^{b}	5.70 ± 0.10
6.0	5.99^{a}	5.99^{a}	5.99^{a}	5.98^{a}	5.97^{a}	5.89^{b}	5.97 ± 0.13
6.4	6.31^{a}	6.31^{a}	6.31^{a}	6.31^{a}	6.30^{a}	6.22^{b}	6.29 ± 0.07
6.8	6.54^{a}	6.56^{a}	6.52^{a}	6.52^{a}	6.51^{a}	6.38^{b}	6.50 ± 0.13
Mean and standard deviation	6.01 ± 0.40	6.01 ± 0.40	6.01 ± 0.40	6.00 ± 0.39	6.00 ± 0.38	5.29 ± 0.38	

[1] n = 20.
[2] Means within a buffer pH followed by the same postscript not significantly different ($P < 0.05$).

Table 5. Analysis of variance of post-incubation pH values, color score and extract release volumes (mean squares).

Source of variation	df	Post-incubation pH		Color		ERV	
		Mean square	Error number	Mean square	Error number	Mean square	Error number
Rigor state (R)	1	1.173**	1	5.80	1	10139.37*	1
Buffer pH (pH)	4	20.670***	2	72.10***	15	440.86***	15
Freeze state (F)	1	0.143**	3	12.62	3	1.88	3
Incubation temperature (T)	5	0.037**	4	80.44***	4	357.79**	4
Animals (A)	4	0.140***	15	25.77***	15	248.69***	15
A × R (Error 1)	4	0.068**	15	19.14**	15	492.92**	15
A × pH (Error 2)	16	0.043***	15	0.77	15	31.20	15
R × pH	4	0.250***	5	1.20	15	307.31**	5
A × F (Error 3)	4	0.008*	15	9.34**	15	69.42*	6
R × F	1	0.114**	15	1.82	6	3.83	15
pH × F	4	0.043	7	0.74	15	190.60**	7
A × T (Error 4)	20	0.022***	15	1.79**	15	82.30**	15
R × T	5	0.002	15	0.49	8	15.98	8
F × T	5	0.002	15	0.55	15	224.51**	15
pH × T	20	0.008*	15	13.37**	15	43.08*	15
A × R × pH (Error 5)	16	0.34***	15	0.99	15	53.08**	15
A × R × F (Error 6)	4	0.004	15	4.26**	15	210.38**	15
A × pH × F (Error 7)	16	0.044***	15	0.69	15	38.35*	15
R × pH × F	4	0.072	11	0.19	11	117.63	11
A × R × T (Error 8)	20	0.010**	15	1.95**	15	40.48*	15
A × pH × T (Error 9)	80	0.004	15	0.74	15	24.52	15
R × pH × T	20	0.005	15	1.42**	15	34.07	15
A × F × T (Error 10)	20	0.004	15	0.78	15	25.96	15
R × F × T	5	0.0009	13	2.05	13	34.24	15
pH × F × T	20	0.0018	15	0.88	15	20.16	15
A × R × pH × F (Error 11)	16	0.037***	15	1.16*	15	52.36**	15
A × R × pH × T (Error 12)	80	0.03	15	0.76	15	24.83	15
A × R × F × T (Error 13)	20	0.0019	15	1.09*	15	17.41	15
A × pH × F × T (Error 14)	80	0.002	15	0.51	15	16.73	15
R × pH × F × T	20	0.003	15	1.20*	15	35.78	15
A × R × pH × F × T (Error 15)	80	0.003		0.59		21.59	15
Total	599						

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 6. Means and standard deviations for color scores and ERV.

Treatment or condition	Color score	ERV
Unfrozen	6.30 ± 1.75	42.36 ± 8.12
Frozen	6.01 ± 1.59	42.47 ± 7.60
Pre-rigor	6.06 ± 1.59	38.31 ± 6.28
Post-rigor	6.25 ± 1.76	46.53 ± 7.19
pH		
5.2	5.20 ± 2.05	44.46 ± 8.24
5.6	5.53 ± 1.79	44.15 ± 8.21
6.0	6.25 ± 1.42	42.46 ± 7.74
6.4	6.83 ± 1.06	40.16 ± 7.04
6.8	6.96 ± 1.09	40.85 ± 7.39
Incubation temp. (°C)		
0	7.07 ± 0.83	43.80 ± 7.99
5	7.01 ± 0.92	43.20 ± 8.54
10	6.72 ± 1.12	44.10 ± 7.12
20	5.65 ± 1.59	43.13 ± 8.07
30	5.56 ± 1.83	41.07 ± 7.81
40	4.90 ± 2.06	39.22 ± 6.80

0.01) each had a significant effect on the post-incubation pH of the homogenates. Significant interactions were found between rigor state × buffer-pH ($P < 0.001$), rigor state × freeze state ($P < 0.01$) and buffer-pH × incubation temperature ($P < 0.05$).

Examination of the means within each treatment level of the significant main effects showed that the post-incubation pH values of pre-rigor tissue were significantly greater than those for post-rigor tissues, and unfrozen homogenates were higher than frozen (Table 3). At all pH levels the mean at 40°C was significantly lower ($P < 0.05$) than those at the remaining temperatures (Table 4). These data do not substantiate results reported in earlier investigations on whole muscle by Briskey *et al.* (1961) and Cook *et al.* (1966a). The magnitude of the pH change during incubation differed for pre- and post-rigor homogenates at the different pH levels (Table 1).

Filtrate color

The mean filtrate color scores at each buffer-pH, incubation temperature, freeze and rigor state are shown in Table 6 and the analysis of variance in Table 5. Incubation temperature ($P < 0.01$) and buffer-pH ($P < 0.001$) each had a highly significant effect together with the interaction, incubation temperature × buffer-pH ($P < 0.01$).

Table 7. Mean color score at each incubation temperature and buffer pH.[1,2]

Buffer pH	Incubation temperature (°C) 0	5	10	20	30	40
5.2	a 7.2X	ab 6.8X	b 6.5X	c 4.4X	c 3.7	d 2.6
5.6	a 7.1X	a 7.2X	b 6.5X	b 4.4X	b 4.7	c 3.4
6.0	a 7.2X	ab 7.0X	b 6.6X	c 5.6	c 5.9	c 5.4
6.4	a 6.9X	a 7.0X	a 7.0X	a 6.7Z	a 6.9Z	a 6.6X
6.8	a 7.0X	a 7.1X	a 7.1X	a 7.2Z	a 6.8Z	a 6.7X

[1] Means within a buffer level followed by the same lower case superscript and those within an incubation temperature treatment followed by the same upper case subscript not significantly different ($P < 0.05$).
[2] $n = 20$.

Analysis of the means by Duncan's multiple range test within each buffer-pH level and temperature treatment is presented in Table 7. The mean color values of the homogenates buffered at the highest levels (pH 6.4, 6.8) did not differ after incubation for 24 hr at the different temperatures. At the remaining pH values (pH 5.2, 5.6, 6.0) filtrate color intensity varied among the incubation temperatures. At these pH values the intensity decreased as the temperature increased.

Color scores were not influenced by buffer-pH when the homogenates were incubated at the lower temperatures (0, 5, 10°C), whereas at the higher temperatures (20, 30, 40°C) the intensity decreased significantly as the pH of the homogenate decreased.

Because some post incubation pH values differed significantly ($P < 0.05$) from the original buffer values, regression and simple correlation coefficients were calculated using actual post incubation data. Table 8 presents the regression equations, t values for regression slopes and simple correlation coefficients between post incubation pH values (X_1) and color score (Y_1) for data within each temperature treatment.

The linear regression provided the best fit for all the data, except that at 30°C, in which the cubic equation substantially reduced the error term (Fig. 1).

Table 8. Relationship between ultimate pH (X_1) and color score (Y_1) and ultimate pH (X_1) and ERV (Y_2) within a temperature treatment.

Incubation temperature (°C)	Color score r	Regression equation	$B_t = B_o$	Extract release volume r	Regression equation	$B_t = B_o$
0	0.08	$Y_1 = 8.02 - 0.15\ X_1$	0.75	−0.45**	$Y_2 = 98.62 - 9.13\ X_1$	5.04**
5	0.05	$Y_1 = 6.26 + 0.13\ X_1$	0.53	−0.37**	$Y_2 = 90.87 - 7.93\ X_1$	3.93**
10	0.24*	$Y_1 = 2.6 + 0.69\ X_1$	2.49*	−0.46**	$Y_2 = 94.01 - 8.31\ X_1$	5.20**
20	0.72**	$Y_1 = -11.91 + 2.93\ X_1$	10.33**	−0.30**	$Y_2 = 80.49 - 6.22\ X_1$	3.15**
30	non linear [1]	$Y_1 = -118.72 + 38.23\ X_1 - 2.88\ X_2 - 0.0047\ X_3$	.....	−0.20*	$Y_2 = 65.41 - 4.05\ X_1$	1.99*
40	0.75**	$Y_1 = -18.99 + 4.03\ X_1$	11.14**	−0.07	$Y_2 = 47.29 - 1.36\ X_1$	−0.76

[1] Cubic form of regression equation $X_1 = pH$, $X_2 = pH^2$, $X_3 = pH^3$.
* $P < 0.05$.
** $P < 0.01$.

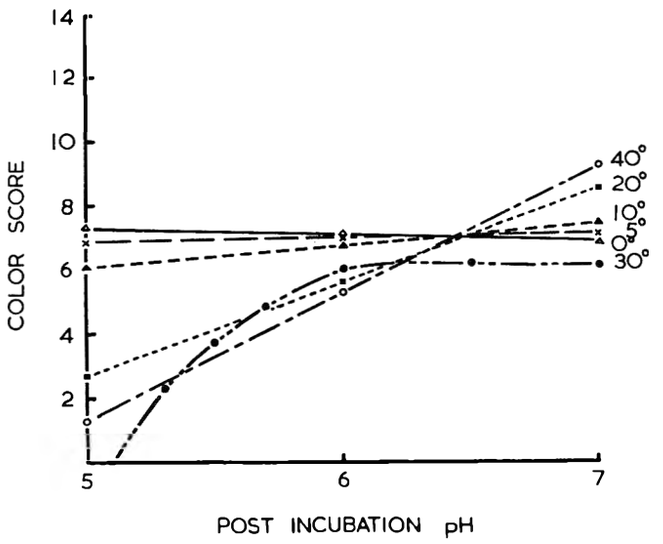

Fig. 1. Regression of ultimate pH (X_1) upon color score (Y_1) at each incubation temperature.

The slopes of the regression lines at the higher temperatures (10, 20 and 40°C) were significantly different from zero, and the correlation coefficients between the two variables were significant, 0.24, 0.72 and 0.77 for the 10, 20 and 40°C samples respectively. At the lower temperatures (0 and 5°C) the relationship was not significant.

These data demonstrate that the color of ovine muscle extract is significantly influenced by pH and incubation temperature, and the combined effect of these two factors. Although variations in pH and incubation temperature manifested significant effects, in each case these differences occur above or below a specific pH or incubation temperature.

These data show that large variations in muscle color are not influenced solely by pH (Winkler, 1939; Munns *et al.*, 1965) but rather by the combined effects of pH and temperature acting concomitantly, (Wismer-Pedersen *et al.*, 1961a; McLaughlin *et al.*, 1963). These two factors brought about color changes of the same magnitude in both pre- and post-rigor homogenates.

Rigor state and freeze treatment had no demonstrable effect upon color development, either individually or in association with other main effects. This suggests that the biochemical manifestations of the development and resolution of rigor, other than decline in pH, do not influence the inherent properties of the muscle which induce or are associated with color development.

Extract release volume

Analysis of variance (Table 5) shows that rigor state of tissue ($P < 0.05$) buffer level of homogenate ($P < 0.001$) and incubation temperature ($P < 0.05$) each had significant effects upon extract release volume. The mean ERV values of the significant main effects are presented in Table 9. The following interactions were significant: incubation temperature × buffer-pH ($P < 0.05$), rigor state × buffer-pH ($P < 0.01$), incubation temperature × freeze state ($P < 0.01$), and freeze state × buffer-pH ($P < 0.01$). These are shown in Tables 9, 10, 11 respectively. The treatment means were analyzed using Duncan's multiple range test.

pH x incubation temperature

Table 9 shows that the ERV of homogenates buffered at the upper pH levels (6.4, 6.8) did not vary among incubation temperatures, but at lower pH values (5.2, 5.6, 6.0) the effect of incubation temperature was significant. Homogenates buffered at 5.2, 5.6 and incubated at 0, 5, 10 and 20°C released significantly larger quantities ($P < 0.05$) of extract than those incubated at the higher temperatures (30, 40°C). At pH 6.0 the ERV of the sample incubated at 40°C was significantly lower than the remainder.

Pooling the data within temperature treatments showed that the ERV of homogenates incubated at the lower temperatures (0, 5, 10 and 20°C) released smaller amounts of extract as the pH was raised. This agrees with the generally accepted postulation indicating a strong relationship between pH and water binding capacity of whole muscle or ERV, (Empey, 1933, Sair *et al.*, 1938; Bouton *et al.*, 1957; Hamm, 1963; Jay, 1964; Cook *et al.*, 1966b). The fact that a similar pattern was not shown for the homogenates incubated at the higher temperatures (30, 40°C) agrees with the drip and cooking loss data on whole muscle (Cook *et al.*, 1966b).

Regression and correlation analyses were calculated between post incubation pH (X_1) and ERV (Y_1) at each temperature treatment. These are presented in Table 8,

Table 9. Mean percent extract release volumes at each buffer-pH and incubation temperature.[1]

Buffer pH	Post-incubation pH	Incubation temperature (°C)[2]					
		0	5	10	20	30	40
		a	a	a	ab	b	c
5.2	5.49	46.75X	46.20X	47.04X	44.94X	42.90X	38.93X
		a	a	a	a	b	b
5.6	5.70	47.06X	44.72X	46.08X	45.82X	40.47X	40.04X
		a	a	a	a	a	b
6.0	5.97	44.05XY	43.96XY	44.52X	42.40YZ	41.39X	38.41X
		a	a	a	a	a	a
6.4	6.29	41.13YZ	39.46Z	41.00Y	40.35Z	40.41X	38.62X
		a	a	a	a	a	a
6.8	6.50	39.99Z	41.63YZ	41.12Y	42.11YZ	40.18X	40.10X

[1] n = 20.

[2] Means within a buffer pH followed by the same lower case superscript and those within an incubation temperature treatment followed by the same upper case subscript are not significantly different ($P < 0.05$).

Table 10. Mean percent ERV for pre- and post-rigor, unfrozen and frozen homogenates at different buffer levels.[1]

Treatment or condition	Buffer pH[2]				
	5.2	5.6	6.0	6.4	6.8
Pre-rigor	a 38.75X	a 39.20X	a 37.80X	a 36.49X	a 39.29X
Post-rigor	c 50.17Y	b 49.11Y	b 47.11Y	a 43.84X	a 42.42X
Difference	11.42	9.91	9.31	7.35	3.13
Unfrozen	a 46.18X	a 44.87X	b 41.75X	b 39.60X	b 39.42X
Frozen	ab 47.73Y	b 43.44X	ab 43.17X	a 40.73X	ab 42.30X
Difference	3.45	1.43	−1.42	−1.13	−2.88

[1] n = 60.
[2] Means within a rigor and freeze treatment followed by the same lower case superscript and those within a buffer pH followed by same upper case subscript not significantly different ($P < 0.05$).

Table 11. Mean percent extract release volumes of frozen and unfrozen homogenates at each incubation temperature.[1]

Treatment of muscle	Incubation temperature (°C)[2]					
	0	5	10	20	30	40
Unfrozen	abc 41.87X	ab 41.66X	d 44.01X	bcd 43.16X	bcd 42.87X	a 40.60X
Frozen	c 45.73Y	bc 44.73Y	bc 44.18X	b 43.10X	a 39.30Y	a 37.84Y

[1] n = 60.
[2] Means within a freeze treatment followed by the same lower case superscript and those within a temperature treatment followed by the same upper case subscript not significantly different ($P < 0.05$).

together with the t values for regression slopes. The linear regression provided the best fit for all the data (Fig. 2). The slopes of all regression lines, except at 40°C, were significant.

Significant negative correlation coefficients between the two variables were observed at all temperatures except 40°C. The highest correlation was at 10°C in which the variation in pH accounted for 21% of the variation in ERV. This decreased as incubation temperatures increased.

Rigor state x buffer-pH

The mean ERV values at each rigor state and buffer-pH are shown in Table 10. At the lower pH levels (5.2, 5.6, 6.0) pre-rigor tissue released significantly smaller amounts of extract than did post-rigor tissue ($P < 0.05$). There was no difference between rigor states for homogenates buffered at pH 6.4 and 6.8. Although it is generally accepted that pre-rigor tissue has greater water binding capacity than post-rigor tissue (Sair *et al.*, 1938; Briskey, 1964; Hamm, 1963) these data show that this relationship between rigor state and ERV is only valid if the pH of the tissue is 6.0 or less.

Since both tissues were subjected to the same buffer level and temperature treatment, it appears that the biochemical changes that occur during the onset and resolution of rigor in whole muscle alter the properties of the proteins in such a manner that at low pH levels (5.2, 5.6, 6.0) their ability to bind water is decreased. It might be assumed that the changes associated with fiber shortening have a significant effect upon water binding capacity. Current investigations strongly substantiate this (Cook, 1968).

Earlier studies (Hamm, 1960; Hamm *et al.*, 1960; Briskey, 1964; Jay, 1964) have demonstrated the existence of a strong positive relationship between muscle water binding capacity and pH values between 5.0 and 11.0. Measurement of ERV shows that this relationship holds true only if the parameters are determined in post-rigor muscle. The data (Table 10) show that variations in buffer-pH did not significantly affect the ERV of pre-rigor muscle.

Freeze state x buffer-pH, freeze state x incubation temperature

Although the freeze state of the tissue had no significant effect upon ERV, the interactions freeze state × buffer-pH and freeze state × incubation temperature were significant ($P < 0.01$) (Tables 10 and 11).

Frozen homogenates incubated at 0, 5°C released significantly greater quantities of extract than the similarly treated unfrozen sample. At 30, 40°C this pattern was reversed. There was no difference between frozen and unfrozen homogenates when incubated at 10°C. The maximum amount of extract was released after incubation at 10°C, and decreased at higher and lower temperatures for unfrozen homogenate. The pattern differed significantly for frozen samples. The ERV was at a maximum at 0°C and gradually decreased to a minimum at 40°C.

The means at each freeze state and buffer-pH are pre-

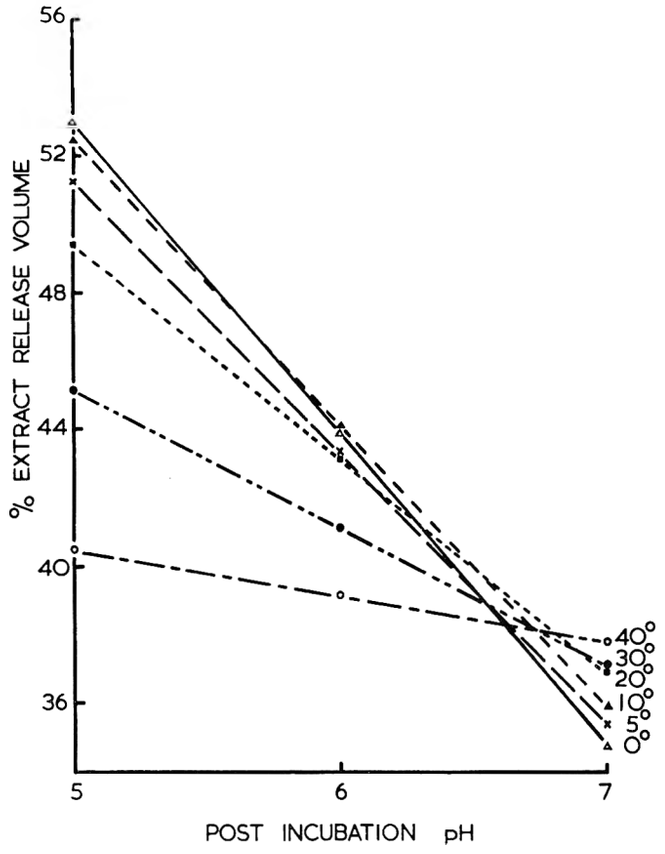

Fig. 2. Regression of ultimate pH (X_1) upon percent extract release volume (Y_1) at each incubation temperature.

sented in Table 10. These data show that the freeze treatment did not affect the ERV of the homogenates buffered at pH 5.6, 6.0, 6.4, 6.8; at pH 5.2 freezing caused a barely significant ($P < 0.05$) decrease. The effect of pH differed among frozen and unfrozen samples. Unfrozen homogenate, buffered at the upper levels (6.0, 6.4, 6.8), released smaller quantities of extract than those buffered at the two lower levels; but when the homogenate was frozen the pattern was altered. The values ranged from a maximum of 43.44% at pH 5.6 to a minimum of 40.73% at pH 6.4.

These data show no definite trends, and any differences that exist are very small. Clearly, these results demonstrate that the major portion of the variation in ERV cannot be accounted for by the individual variations in freeze state, rigor state, pH and incubation temperature, but rather by the interaction of these factors acting concomitantly.

Further investigation of these combined factors on the biophysical properties of the proteins is needed in order that a meaningful interpretation of the influence of these factors on ERV may be attempted.

Special attention should be paid to the interpretation of these data. Techniques for determining the moisture binding capacity of muscle, filter press, centrifugation, drip loss, cooking loss and ERV may measure different parameters; thus a direct comparison of data obtained by these methods should be done with caution.

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Stepwise Discriminant Analysis of Gas Chromatographic Data as an Aid in Classifying the Flavor Quality of Foods

SUMMARY—Stepwise discriminant analysis for classifying food samples (known independently to differ in flavor) is illustrated by computer analysis of gas chromatograms from roasted coffee and potato chips. Four lots of coffee prepared so as to differ in flavor were scored organoleptically, steam distilled, and the distillate examined gas chromatographically. By calculating all possible ratios among peak heights and subjecting these ratios to discriminant analysis, the coffee could be classified into the four flavor categories from the gas chromatographic data. The discriminant analysis procedure was set to select the ratio most critical in differentiating among the chromatograms, then move on to the next most efficient ratio until the samples were classified. The same thing was done for potato chips except headspace volatiles were used for the gas chromatographic analysis.

Not only does the procedure described enable flavor to be correlated with gas chromatographic data, but the efficiency values for each ratio are useful. A compound devoid of flavor can conceivably be highly correlated with flavor; however, there is a good chance that a compound highly correlated with flavor is a flavor substance itself.

INTRODUCTION

THE UTILIZATION OF GAS chromatographic data has often not kept pace with its acquisition. This is probably more true in flavor evaluation than elsewhere. Visual inspection or even simple mathematical evaluation of a gas chromatogram is often fruitless in attempting to relate organoleptic flavor to chemical change. Flavor deterioration frequently results from subtle changes in several components rather than drastic changes in a relatively few compounds. Means need to be developed to accumulate the effects of small changes in flavor components. The objective of this study was to learn whether discriminant statistical analysis would permit a classification of foods by flavor from gas chromatographic data.

REVIEW OF LITERATURE

NUMEROUS INVESTIGATORS have observed gas chromatographic differences in the volatiles of foods known to differ in quality or processing method. Typical of such observations are comparisons of brewed coffee and coffee in the can, fresh and imitation banana, and good- and poor-quality bourbon (Mackay *et al.*, 1961); different coffee samples (Rhodes, 1958); two varieties of bananas at four stages of maturity (McCarthy *et al.*, 1963); alcoholic beverages of different types (Bober *et al.*, 1963, 1964); turkey and chicken products (Pippen *et al.*, 1963); three varieties of oranges (Wolford *et al.*, 1963); fresh and canned corn, fresh and off-flavor potato granules, and two varieties of pear (Buttery *et al.*, 1961); and age of dry-cured hams (Ockerman *et al.*, 1964).

Coffman *et al.* (1960) suggested that gas chromatographic profiles could be used as a standard for quality control purposes. Buttery *et al.* (1963) concluded that chromatographic analysis could help resolve sources of off-flavor where the compounds arose from several different types of food deterioration. Jennings *et al.* (1962) had concluded likewise.

Most attempts to correlate gas chromatographic data with organoleptic flavor have not involved use of the entire chromatograms but rather one peak, or relatively few peaks, among all those detected (Boggs *et al.*, 1964; Bengtsson *et al.*, 1964; Dornseifer *et al.*, 1963, 1965; Weurman, 1961). This is satisfactory if a good correlation exists between flavor and a few chemical compounds. Boggs *et al.* (1964) found that the hexanal content in the vapor above potato granules was a good index of rancidity though it was not the sole cause of it. Vorbeck *et al.* (1961) observed that formation of a cheese-like off-flavor in sauerkraut was related to the formation of abnormally large amounts of three fatty acids. Often, however, correlation has been poor (Dimick *et al.*, 1956; Fernandez, 1963; and National Canners Association, 1966).

Rohan (1965) used the ratios between two peaks to assess the chocolate aroma potential of unroasted cocoa beans. Kayahara *et al.* (1966) used the ratios between isoamyl, isobutyl, and propyl alcohols to differentiate Scotch from Japanese whiskey. Jennings *et al.* (1960) smelled each peak as it emerged from the column and reported that the higher-molecular-weight components were more important to flavor than the first compounds to emerge.

Guadagni *et al.* (1966) used a technique similar to that of Jennings *et al.* (1960) with an added innovation. They determined the threshold value of each compound and recommended that the peak area be weighted by the threshold value in deciding the importance of each compound as a flavor substance.

Stewart (1963), Burr (1964), Wick (1965), and Patton *et al.* (1966) have discussed some of the problems of relating gas chromatographic data to organoleptic flavor response.

Lawson *et al.* (1963) used a Fortan source program for analysis of gas chromatographic retention times, and Flynn *et al.* (1965) have suggested procedures for computer interpretation of gas chromatograms.

EXPERIMENTAL

THE DISCRIMINANT-ANALYSIS PROCEDURE used was a stepwise one prepared by the Health Sciences Computing Facility, University of California at Los Angeles (Biomedical Computer Programs, 1964). It was already on hand in the program bank at the University of Georgia Computer Center. The program is set to select the variable of greatest discriminatory value and use it for making a classification. The next most efficient variable is then selected, and the samples are classified anew. This system is followed until all the variables have been used.

In arriving at classifications based on cumulative differences, the stepwise-analysis procedure in effect is also ranking the variables for efficiency as discriminatory factors. The program is written so that the order of the variables is selected according to: (1) the variable with the largest F value; and (2) the variable which when partialed on the previously entered variables has the highest multiple correlation with the groups; and (3) the variable which gives the greatest decrease in the ratio of within-group to total variation.

Kendall (1961) and Goulden (1952) have described the function and calculation of various types of discriminant analyses. Jennrich *et al.* (1965) have described the particular stepwise discriminant analysis procedure used here.

It was decided that all possible ratios among the gas chromatographic peak heights (or areas) might reflect cumulative differences more than anything else. The program was written to handle 80 variables. Whenever the number of chromatographic peaks exceeded nine, the number of variables (ratios) was thus beyond the capacity of the program. In that case, the data were sub-divided and stepwise calculations were carried out on the subclasses instead of all the data as a unit.

The discriminant analysis procedure was first applied to 24 chromatograms for coffee. Four batches of roasted coffee were replicated twice as to preparation of batches and in triplicate as to analysis. The chromatograms were furnished by a commercial coffee firm. These chromatograms were used because they were fairly complex (50–66 peaks) and the quality of the coffee had been evaluated organoleptically by experts, also the data were essentially unknowns to the authors. It was desired to learn whether the same peaks would be picked by discriminant analysis as being correlated with flavor as had the coffee company from experience by visual inspection and simple mathematical calculation.

One variety of coffee was used for Batch A. The roasted coffee made from this variety was scored excellent in flavor. Another variety was used for Batch D. This coffee was acceptable in flavor. Batch B consisted of a 60/40 blend of Batches A and D. Batch C consisted of a 40/60 blend of Batches A and D. The chromatograms were obtained by steam distilling 20 g of roasted coffee until 40 ml of distillate were obtained. A Triton X-305 column with the temperature programmed from 60 to 210°C was used, and 25 μl of distillate were injected.

Six replicate chromatograms were furnished for each of the four lots. Actually, the six replicate determinations were secured in two sets of triplicate analyses made two months apart. Although the corresponding gas chromatograms showed the same over-all profile, the peak heights for the second set of three were 16–23% less. Changes in the column, instrument sensitivity, and other factors well known to gas chromatographers—though not always well understood—probably caused the discrepancy between the two sets of data. Without correction this would have increased experimental error and made discrimination more difficult. To overcome this problem, each peak height was calculated as the percentage of the total for all peak heights on a given chromatogram; thus, the second set of triplicate values, though smaller, was put on the same basis as the first set. In other words, the sum of all the peak heights was used as a reference to compensate for instrument variability.

After details of the stepwise procedure using the chromatograms for coffee had been worked out, the procedure was recommended to Miller (1966) who had been studying other means of discriminant analysis for gas chromatographic profiles. One set of Miller's data will be used to illustrate a limitation of stepwise discriminant analysis when only relatively simple chromatograms are available. Miller (1966) aged potato chips in the dark at 23°C and examined them organoleptically and gas chromatographically at intervals up to five weeks.

The organoleptic procedure was the same as that described by Dornseifer *et al.* (1965) based on the two-sample difference test outlined by Ellis (1961). Miller (1966) showed that chips stored at 23°C in the dark were judged to be off-flavor in 22 days at the 0.05 level of significance and in 31 days at the 0.01 level of significance. The gas chromatographic columns used were ¼-in. copper tubing, 12 ft long, packed either with 10% LAC-446 (polyethylene glycol adipate polyester) or with 10% Carbowax 20–M on 60–80-mesh Chromosorb W. The headspace sample size injected was 1 ml.

RESULTS AND DISCUSSION

STEPWISE DISCRIMINANT ANALYSIS of the chromatograms for the four coffee samples revealed that often one ratio alone was sufficient to group the coffee into four distinct flavor categories and rarely were more than four ratios required. Representative chromatograms for the four lots of coffee are shown in Fig. 1. Table 1 shows the results from a small portion of a stepwise analysis. When Peaks 1 and 2 were used as denominators, only

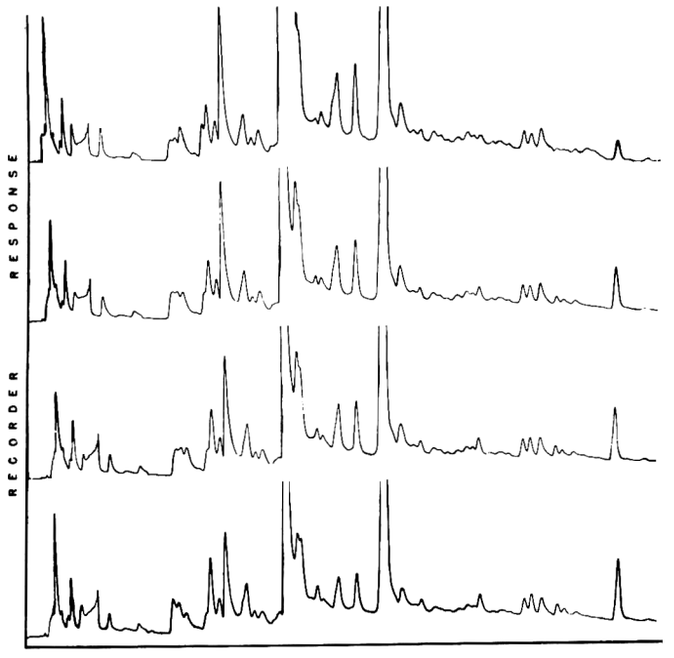

Fig. 1. Reading downward, the chromatograms represent the distillates of Batches A, B, C, and D of the roasted coffee.

four ratios were needed to classify the coffee correctly. In the top part of the table, the first four steps are listed. The ratio between Peaks 32/2 was most effective as a discriminating factor.

The U-statistic reflects the closeness to which the classification was coming to perfection. In the center of the table, the classification is given based on the first step, or the ratio between Peaks 32/2. The differences among the ratios for the four treatments were insufficient to classify the batches properly. Step 1 shows that Batches A, B, and C were properly classified, but one sample of Batch D was incorrectly classified as a Batch C sample. Though the classification was coming closer as judged by the U-statistic when Steps 2 and 3 were used as further discriminatory factors, the cumulative differences were still insufficient to change the classification. By the fourth step the cumulative effects of differences among treatments were sufficient to enable the four batches to be properly classified. This is shown at the bottom of the table.

Table 1. Results of stepwise discriminant analysis for 4 most efficient ratios involving Peaks No. 1 and 2 as denominators.

Step	Variable	Peaks used to form ratio	F value to enter or remove	U-statistic
1	66	32/2	169.9745	0.0377
2	40	8/2	8.8025	0.0158
3	37	5/2	6.4799	0.0076
4	41	9/2	6.4414	0.0036

	Predicted batch (6 replicates)			
Actual batch	A	B	C	D
	Step 1			
A	6	0	0	0
B	0	6	0	0
C	0	0	6	0
D	0	0	1	5
	Step 4			
A	6	0	0	0
B	0	6	0	0
C	0	0	6	0
D	0	0	0	6

Table 2 shows other ratios which were effective discriminatory factors. When Peaks 3 and 5 were the denominators, two ratios were needed to effect classification. Peak 7 divided into Peak 32 was adequate by itself. Table 2 shows that usually 1 to 2 ratios among the 66 in each subset were sufficient to yield correct classification. However, the ratios involving Peaks 14 and 15, 31.2 and 31.3, and 32 as denominators were almost worthless for classification purposes.

The reason relatively few sets of ratios were needed to classify the batches properly may be seen in Table 3. This table shows the same ratio combinations as Table 2 except here the mean ratios themselves are listed. In general, the ratio increased (or decreased) steadily with flavor change. The ratio between Peaks 32/2 became progressively larger the poorer the quality. The ratio between Peaks 9/2 became progressively smaller. Though none of the ratios which changed curvilinearly with treatment were adequate by themselves to differentiate among the treatments (as might be expected), they were useful. The ratios of Peaks 9/2 were curvilinear with treatment; yet this ratio was the second most efficient ratio when Peaks 1 and 2 were denominators.

The 33 peaks which were employed to form the ratios were used to compare discriminant analysis predictions with the coffee company's experience. There was fair agreement as to which peaks were most useful. Originally, it was intended to analyze the ratios generated from 33

Table 2. Ratios between coffee gas chromatographic peaks which correctly classified batches.

Peaks used as denominators versus all other peaks	Ratios[1] between peaks necessary for correct classification
1, 2	32/2, 8/2, 5/2, 9/2
3, 5	32/5, 9/5
6, 7	32/7
8, 9	32/9, 24/9
10, 11	19/11, 32/11
12, 13	22/13
14, 15	8/14, 29/14, 19/15, 6/14, 28/15, 19/15, 28.6/14, 10/14, 3/15, 21/15, 19/14, 7/15, 31.3/14, 22/14
16, 17	24/16
18, 19	32/18
20, 21	32/21
22, 23	3/22, 22/23
24, 26	16/24
27, 28	32/28, 21/28
28.6, 29	19/28.6
30, 31	32/31
31.2, 31.3	13/31.3, 3/31.3, 29/31.3, 12/31.3, 31.2/31.3, 22/31.3, 6/31.3, 5/31.3, 19/31.3
32	No ratio properly classified batches

[1] The first ratio listed is most efficient in discriminating value, and all ratios thereafter are in order of decreasing efficiency as discriminating factors.

peaks, then study the ratios from the remaining peaks in case the ratios from the 33 peaks were insufficient to permit discrimination among batches. Had ratios been generated from the remaining peaks, perhaps other ratios might have been found such as 32/7, 22/13, or 24/16 where one ratio alone was sufficient to classify the food for flavor.

In practice, the more useful ratios among these and the 22 useful ratios listed in Table 3 could be combined and subjected to stepwise discriminant analysis to rank the most useful ratios in the order of efficiency. This was not done because it was not necessary. The cumulative effect from several ratios was not needed to get proper classification.

To illustrate a limitation of stepwise discriminant analysis, a classification applied to Miller's data is shown in Table 4. Typical chromatograms are shown in Fig. 2. From Table 4, one may see that the classification was poor after the first step of calculation. Table 4 shows that under Step 1, for example, three of the replicates were correctly identified as being 1 week old, but three were incorrectly classified as being 2 weeks old. By the fourth calculation, Step 4, the classification had improved somewhat. By the ninth step it was almost perfect, but not until the 19th ratio had been used were the chips correctly classified by age from the gas chromatographic profiles. Table 5 shows the mean values by age for the ratios and the order of ratios according to efficiency.

One thing is evident in comparing Tables 3 and 5. With few exceptions, the ratios used in classifying the coffee tended to increase (or decrease) progressively as flavor scores decreased. Nearly all the ratios in Table 5 are curvilinear with age. The 22 ratios listed in Table 3 were drawn from among 1,056 possible ratios; the 19 ratios in Table 5 were drawn from among 42 possible ratios. The more peaks there are, the greater is the possibility of finding peaks (ratios) highly correlated with flavor change, and ratios which change progressively in one direction or the other as quality changes.

The fact that some of the ratios were curvilinear is not surprising. Peroxide values increase initially, then decrease as fat rancidification progresses. The National Canners Association (1965) reported that the carbonyl content of olives was below 2 ppm when the flavor was good but rose to 2.5 to 17 ppm as flavor deteriorated. Upon further investigation, it was concluded (National Canners Assoc., 1966) that the amount of carbonyls present was not a good index of off-flavor. The carbonyl content increased, then decreased as deterioration progressed; consequently, one would have to know the course of change as well as the amount of carbonyls present if carbonyl content alone were to be the objective index of deterioration. The data in Table 5 suggest that curvilinear values can be used as indices of change if there are enough values to permit accumulation of differences according to treatment.

One possibility to improve the degree of classification of the potato chips might have been to insert intensity factors so as to weigh peak areas as recommended by Guadagni *et al.* (1966). However, this might not lead to better classification. There is much literature indicating that the intensity of odor characteristics of a compound is different in mixtures than as an individual compound. Guadagni *et al.* (1963) found, for example, that subthreshold concentrations of some compounds produced an additive effect. Kendall *et al.* (1964) observed that the nose judged the odor of compounds emerging from the gas

Table 3. Mean ratios between various peaks for the four batches of coffee.

Ratio	Batch A	Batch B	Batch C	Batch D
32/2	0.522	1.352	1.912	2.552
8/2	0.762	0.677	0.659	0.820
5/2	0.763	0.823	0.942	1.039
9/2	0.828	0.691	0.664	0.520
32/5	0.690	1.673	2.043	2.495
9/5	1.088	0.861	0.719	0.519
32.7	0.318	0.774	0.958	1.273
32/9	0.635	1.966	2.887	4.988
24/9	1.974	2.315	2.464	2.594
19/11	6.994	4.774	3.769	2.494
32/11	0.810	1.541	1.814	1.985
22/13	0.189	0.464	0.687	1.306
24/16	1.463	1.131	0.991	0.733
32/18	0.874	2.409	3.516	6.015
32/21	0.156	0.405	0.572	0.830
3/22	19.753	9.353	7.227	5.487
22/23	0.474	1.169	1.693	3.051
16/24	0.684	0.885	1.010	1.367
32/28	0.708	1.740	2.328	4.324
21/28	4.546	4.289	4.126	4.300
19/28.6	113.053	53.206	33.228	14.780
32/21	0.829	2.003	2.637	3.773

Table 4. Classification of potato chips by age after steps 1, 4, 9–18, and 19 of stepwise discriminant analysis.

Actual age of chips in weeks	Predicted age of chips				
	One	Two	Three	Four	Five
			Step 1		
1	3	3	0	0	0
2	2	2	2	0	0
3	1	1	4	0	0
4	0	0	1	2	3
5	0	0	0	3	3
			Step 4		
1	3	3	0	0	0
2	0	6	0	0	0
3	0	0	6	0	0
4	0	0	1	5	0
5	0	0	0	0	6
			Steps 9 to 18		
1	5	1	0	0	0
2	0	6	0	0	0
3	0	0	6	0	0
4	0	0	0	6	0
5	0	0	0	0	6
			Step 19		
1	6	0	0	0	0
2	0	6	0	0	0
3	0	0	6	0	0
4	0	0	0	6	0
5	0	0	0	0	6

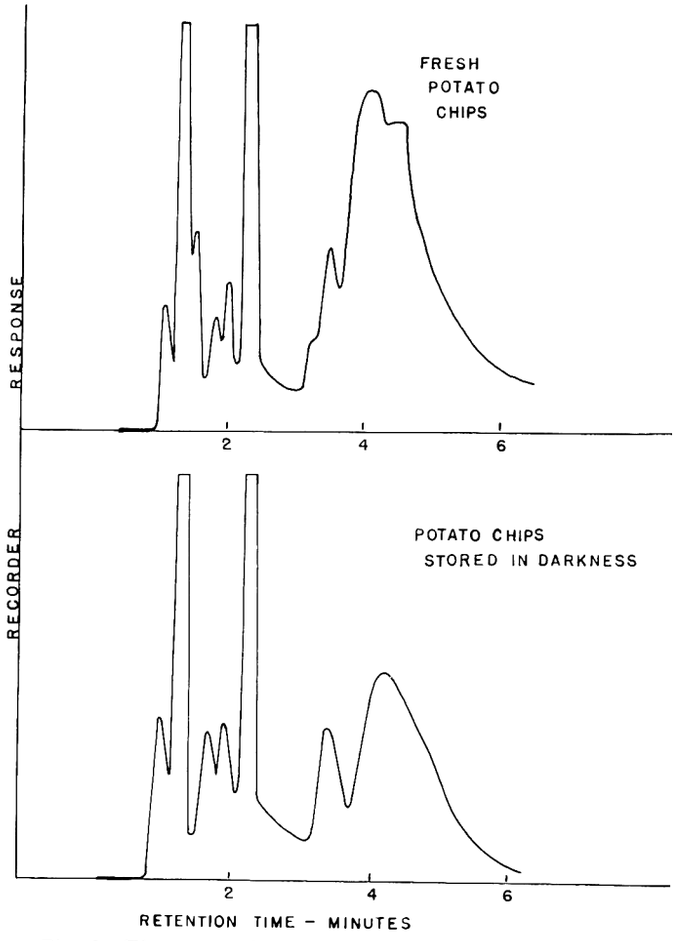

Fig. 2. The upper chromatogram shows a typical profile for the headspace volatiles of fresh potato chips. The lower chromatogram shows a profile for potato chips stored 22 days in darkness at 23°C.

chromatograph column by the traces of contaminants as well as the main compound.

Meijboom (1964) found a masking effect occurs in odor or taste in certain ratios of volatiles. Bennett *et al.* (1965) likewise observed that the presence of a second compound often affects threshold values. Langler *et al.* (1964) reported that certain ketones exhibited a synergistic interaction whereby a perceptible flavor became evident though the concentrations of all the compounds were below their respective threshold values. Kendall *et al.* (1966) have listed some of the types of interaction they found.

Even if intensity factors were useful, there is a further problem. A great deal of work would be required to trap peaks and determine the threshold value for each compound.

One disadvantage to the particular discriminant-analysis procedure used is that all the gas chromatographic data was not utilized. Only peaks which appeared in all the chromatograms of all the treatments could be used because the computer program was designed to handle only continuous variables. If a peak appeared or disappeared, it was not used. This did not matter with the data for the coffee because several of the ratios were so discriminatory in their own right that extensive accumulation of differences was not necessary.

For the potato chip data, the use of all peaks would have been desirable. With the particular Program used, the only way to accomplish this would have been to arbitrarily insert an infinitesimally small value for a peak when it was lacking in a given treatment. In this way a ratio could have been generated. A better way would be to have a computer program written which makes provision for peaks arising or disappearing. Shortly after this study was conducted, Bryan (1966) prepared a Program which makes provision for continuous, categorical, and rank variables. The Program provides for including one

Table 5. Mean ratios of gas chromatographic peaks from potato chips aged in the dark at 23°.

Step number	Peak ratios	Age of chips in weeks and mean values for ratios (6 replicates)				
		One week	Two weeks	Three weeks	Four weeks	Five weeks
1	7/1	1.665	1.756	2.223	4.026	4.557
2	9/6	1.465	1.379	0.957	1.169	0.996
3	7/2	1.000	1.000	1.000	1.118	0.948
4	9/5	1.499	1.701	1.522	1.576	1.451
5	5/9	0.677	0.590	0.659	0.725	0.692
6	9/7	0.676	0.750	0.429	0.312	0.251
7	6/2	0.495	0.543	0.415	0.272	0.242
8	2/6	2.509	1.884	2.338	3.812	4.195
9	5/2	0.445	0.440	0.282	0.201	0.167
10	9/10	0.731	1.007	0.956	0.968	0.613
11	6/10	0.538	0.730	1.014	0.773	0.621
12	10/2	0.924	0.783	0.505	0.386	0.391
13	1/9	0.970	0.808	1.096	1.314	0.914
14	5/6	0.998	0.813	0.631	0.746	0.693
15	5/1	0.713	0.766	0.612	0.772	0.798
16	1/10	0.672	0.777	1.019	0.778	0.552
17	1/2	0.616	0.594	0.462	0.298	0.215
18	7/10	1.092	1.356	2.325	3.016	2.441
19	10/5	2.211	1.777	1.826	2.014	2.412

type of variable or all three, but, unfortunately, it cannot be used for only two types of variables.

Another consideration which needs to be taken into account is dual use of ratios involving the same peaks. In Table 5, for example, Step No. 4 was the ratio of Peaks 9/5 whereas Step No. 5 was the reverse, Peaks 5/9. The program should be set to discard a ratio if its reciprocal has already been used in the step progression; this in effect is using the same data twice. The same thing shows up in Steps 7 and 8, Table 5. However, reciprocal ratios are not always of nearly equivalent discriminatory value. In Table 2 Peak 32 was often a numerator in discriminating ratios. In fact, it shows up nine times as a numerator; yet as a denominator the cumulative effect of the 32 ratios involving it was insufficient to classify the four blends correctly.

Means other than the one used to correct for instrumental variation such as encountered with the coffee data might be used. When variability between treatments known to be alike is encountered, an internal reference (Kepner *et al.*, 1964) might be used.

Ideally, the gas chromatographic areas should be integrated and fed directly onto magnetic tape. In turn, the magnetic tape could be fed to a high-speed computer to avoid manual manipulations or transferring of data.

For flavor studies, discriminant analysis at best will complement organoleptic evaluation, not replace it. It does have the advantage, however, that more objective comparisons can be made by food companies having production plants at several locations than by using sensory panels in different locations, because variability among panels is not uncommon. For maximum advantage, the program should be written to handle discontinuous as well as continuous variables.

Stepwise discriminant analysis could also be put to good use in identification studies. Because the stepwise progression ranks the various ratios according to their correlation with flavor, the rankings—among other criteria—might be used to decide which compounds should be identified first when identity is not known. Sometimes compounds highly correlated with flavor will not be flavor substances. More commonly they will.

Of the 103 compounds listed by Gianturco (1967) as being components of coffee aroma, for example, only a few would be considered to be of low odor intensity. The 33 peaks used in the trials here were scattered throughout the chromatogram so that they represented small peaks, large peaks, low-boilers, and high-boilers. Some of the peaks in ratios highly correlated with flavor were small peaks. Discriminant analysis, among other means, would alert one to the fact that these peaks should be among the first to be studied.

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Determination of Flavor Threshold Levels and Sub-Threshold, Additive, and Concentration Effects

SUMMARY—The flavor threshold concentrations were determined for 23 compounds composing an artificial peach beverage base. The threshold levels ranged from 52 ppm to 0.4 ppb. The threshold values were used in sub-threshold, additive sub-threshold, and concentration trials. Sub-threshold and additive sub-threshold effects resulted from only a few of the flavor combinations tried. Change in concentration of one compound in a mixture of six compounds was not readily detectable organoleptically. The difference in concentration could be detected gas chromatographically, but only if the mixtures were extracted with pentane and concentrated.

By dividing the respective threshold values into the amount of each compound present in the beverage, a factor was calculated, called the "unit flavor base," which indicated the relative importance of each compound as a flavor substance in the peach beverage. By dividing the gas chromatographic peak heights by the respective threshold values, gas chromatographic response was weighted for flavor importance. When the unit flavor base and weighted gas chromatographic response were converted into logarithms, the relation between the values was nearly linear. The correlation coefficient was 0.8691 (significant @ 0.01).

INTRODUCTION

POWERS ET AL. (1968) described a discriminant analysis procedure to relate gas chromatographic patterns to the flavor of a food. While the method described worked well for coffee samples, there are some theoretical disadvantages which need to be examined. The gas chromatograph senses compounds by separating them. Human beings evaluate flavor by subconsciously integrating their sense response to the different substances present.

In addition, the nose as a sensing device usually is more sensitive to flavor substances than mechanical detectors. Furthermore, there may be organoleptic interactions among compounds; the presense of one compound may depress (or heighten) sensitivity to another compound. The purpose of this study was to determine flavor threshold values and to examine combinations of flavors for organoleptic interactions.

REVIEW OF THE LITERATURE

Amerine *et al.* (1965) cite several studies dealing with threshold levels and interactions among taste substances,

a lesser number of reports on odor studies, and comparatively few on threshold and interaction trials for aqueous solutions of volatile substances. Typical of those on taste compounds are the study of Fabian *et al.* (1943) who observed competitive and compensatory action and those of Pangborn (1960, 1962) who reported interactions among compounds at sub-threshold, threshold, and supra-threshold levels.

Organoleptic interactions among substances are not confined to taste substances. Sjostrom *et al.* (1957), Bartley (1958), and Kendall *et al.* (1966) described interactions among odor substances. Meijboom (1964) observed that volatiles in certain ratios may mask the odor of other compounds. Moncrieff (1951) cites several examples of "masking" by odor substances.

Berg *et al.* (1955a) showed that there were organoleptic interactions between non-volatile and volatile substances. Hinreiner *et al.* (1955a,b) observed that threshold levels were affected by the presence of other compounds whether volatile or non-volatile and that threshold levels varied according to the solvent (wine or water).

These organoleptic interactions among volatile and non-volatile substances have practical application. Singleton *et al.* (1962) found, for example, that blended wines were more organoleptically acceptable the more complex the blend. Berg *et al.* (1955b) measured threshold values of some volatile compounds in aqueous solution. As between volatile compounds, Bennett *et al.* (1965) showed that the presence of other volatiles in fermented dairy products affected perception of diacetyl.

Lillard *et al.* (1961) found that carbonyls were additive in their effect at marginal or sub-threshold levels. Day *et al.* (1963) and Guadagni *et al.* (1963) reported that mixtures of carbonyls at subthreshold levels were additive. Guadagni *et al.* (1963) found that the same thing was true for a mixture containing an alcohol, an acid, a sulphide, an aldehyde and an amine. Langer *et al.* (1964) reported that certain ketones exhibited synergistic interaction whereby a perceptible flavor became evident though the concentration of each compound was below its respective threshold level.

Lockhart *et al.* (1950) found, on the other hand, that subliminal amounts of monosodium glutamate had no effect on the taste of supraliminal amounts of sucrose or sodium chloride. Kendall *et al.* (1964, 1966) observed that the nose judged the odor of compounds emerging from a gas chromatograph by their trace components as well as the major component and observed that the nose is usually more sensitive than gas chromatographic detectors. Guadagni *et al.* (1966) proposed that peak areas of gas chromatograms be weighted by the threshold value in deciding upon the importance of each compound as a flavor component in foods.

METHODS

Materials

The flavor compounds used were furnished by International Flavors and Fragrances. They were selected to compose an artificial peach base. Tepyl acetate (1–3 nonanediol acetate mixed esters), para-tolyl aldehyde, and acetaldehyde were also components of the peach base, but these compounds were not used in the study because the first two contained more than one component and the acetaldehyde was dissolved in alcohol.

Twenty ft ¼″ stainless steel columns with 10% Triton X-305 coated onto 60–80 mesh Diatoport S were used in a dual-column gas chromatograph with flame detectors. The temperature was programmed from 65–200° at 2°/min, then held at 200°.

Taste panel

The taste panel initially consisted of 28 individuals who were either students or staff members. The panel was divided about evenly as to sex, and the panelists ranged in age from approximately 18 to 55 years of age. The number of tasters decreased during the study from 28 to 22 panelists because some of the members graduated. Panelists were selected for their consistency, but not particularly for acuity, since levels detectable by the average person were sought.

A triangular test was used for all taste panels. The order of presentation of samples was varied so that the panel members were not tasting the distilled water and test solution in the same order. Whether there were two glasses of distilled water and one of test solution were also reversed at random. All results were determined at the 0.05 level of significance.

Threshold trials

In the threshold trials each of the 23 compounds was first presented to the panel at the concentration it would be present in an artificial peach beverage. If the compound was detectable at this level, it was tested at 90, 80, 70, 60, 50, 40, 30, 20, 1, 0.1, 0.01, and 0.001% of beverage strength until a level was reached where the compound was no longer detectable.

The level called the threshold was that percentage of the beverage concentration where a significant number of tasters could detect the compound whereas the next lowest level of the compound was non-detectable. If the compound could not be detected at 100% of its beverage strength, then the concentration was increased at 10% intervals until a significant number of panelists could detect the compound.

Sub-threshold effects

Six compounds were used to test for sub-threshold effects. To be sure that threshold effects were not actually being measured, a concentration two levels below the threshold was used. For example, the threshold of benzaldehyde was 30% of that found in the peach beverage; therefore, when testing for sub-threshold effects benzaldehyde was used at 10% of beverage concentration.

The compound tested was used with two or more compounds at their threshold levels. In one (or two) of the three tasting glasses, all three compounds were present; in the other glass(es) only the two compounds at their threshold levels were present. From two to five compounds at their threshold levels were used in conjunction with the compound at the sub-threshold level (Table 1).

Additive sub-threshold effects

To determine if sub-threshold additive effects could be detected, first two compounds were added to distilled water

Table 1. Combinations used to test for sub-threshold effects.

Substance at sub-threshold level	Substances at their respective threshold levels	
Isovaleric aldehyde (33% of threshold level)	*Benzaldehyde* *2-methyl butyric acid*	Benzaldehyde gamma-undecalactone
	Ethyl cinnamate Ethyl acetoacetate	gamma-undecalactone Ethyl cinnamate
	2-methyl butyric acid Ethyl cinnamate gamma-undecalactone	Ethyl acetoacetate Ethyl cinnamate gamma-undecalactone
	Ethyl acetoacetate Ethyl cinnamate gamma-undecalactone Benzaldehyde	Benzaldehyde 2-methyl butyric acid
2-methyl butyric acid (92% of threshold level)	Benzaldehyde Isovaleric aldehyde	
Ethyl acetoacetate (92% of threshold level)	Isovaleric aldehyde Benzaldehyde	Benzaldehyde gamma-undecalactone
	Ethyl cinnamate gamma-undecalactone	2-methyl butyric acid Isovaleric aldehyde
	Isovaleric aldehyde gamma-undecalactone	2-methyl butyric acid gamma-undecalactone
Benzaldehyde (33% of threshold level)	gamma-undecalactone Ethyl cinnamate	2-methyl butyric acid Isovaleric aldehyde
Gamma-undecalactone (1% of threshold level)	Benzaldehyde Isovaleric aldehyde	
Ethyl cinnamate (1% of threshold level)	Ethyl acetoacetate gamma-undecalactone	Benzaldehyde Isovaleric aldehyde

below their threshold level, then three compounds, then four, etc., until a total of eight compounds were used. Again, the level of compound used was set two increments below the level determined to be the threshold. This resulted in compounds being tested at different percentages of their respective threshold levels (Table 2). Trials were also made in which the sub-threshold concentration was set at a constant percentage of the threshold. The sub-threshold level picked was 80% of the respective threshold concentration of each compound.

Concentration effects

Trials were made to determine if the panel could detect a change in concentration of one compound in a mixture. The compound, the concentration of which was being varied, was added at 1% and 100% of its strength in the finished beverage with five other compounds which were at their beverage level. A second series of trials was also conducted with the five compounds at their respective threshold levels and the sixth compound again being at 1% and 100% of its beverage level.

Table 2. Compounds and amount of compounds used in sub-threshold and additive threshold trials.

Compound	Threshold (ppm)	Sub-threshold amt (ppm)	% Sub-threshold was of threshold
Ethyl acetoacetate	0.52	0.48	92
Ethyl cinnamate	0.016	0.00016	1
Benzaldehyde	1.53	0.51	33
2-methyl butyric acid	1.58	1.45	92
Gamma-undecalactone	0.15	0.0015	1
Isovaleric aldehyde	0.165	0.055	33
Vanillin [1]	0.68	0.0068	1
Amyl butyrate [1]	1.3	0.068	5.2

[1] Were used only in the additive sub-threshold trials.

Gas chromatographic analysis

Four gal of each of the solutions previously described under concentration effects were extracted with 500 ml of pentane four times. The pentane was concentrated in a Rota-evaporator to a final volume of 20 ml. Fifty ml of the concentrated solution were used for gas chromatographic analysis.

Another gas chromatographic phase involved the injection of 1 ml of each of the 23 compounds used in the organoleptic phases. The peak height of each compound was measured and used to calculate a factor which related organoleptic and gas chromatographic analysis. This factor, called "weighted peak height," was obtained by dividing the peak height by the threshold.

RESULTS AND DISCUSSION

THE FLAVOR THRESHOLD LEVELS of the 23 compounds examined varied from 52 ppm for ethyl alcohol to 0.4 ppb for ionone alpha white (Table 3). The threshold values were then used to calculate the relative importance of the different compounds in the peach base. The lower the threshold value, the more intense is the flavor; accordingly by dividing the threshold value into the

Table 3. Threshold levels, unit flavor base, and weighted peak heights.

Compound	Threshold ppm	Amt in finished beverage g/gal	Amt in finished beverage ppm	Unit flavor basis (ppm in beverage/ threshold ppm)	Retention time (min)	Peak height (cm)	Weighted peak height (peak height/ ppm threshold)
Ethyl alcohol	53.0	.....	....	....	....	....	....
Maltol	7.1	0.0027	0.71	0.1	80.4	13.7	1.9
Benzyl alcohol	5.5	0.0104	2.75	0.5	50.6	12.7	2.3
Amyl valerate	4.7	0.0357	9.43	2.01	24.2	14.5	3.1
Ethyl acetate	3.0	0.0372	9.83	3.28	6.0	16.8	5.6
2-methyl butyric acid	1.6	0.0024	0.63	0.31	40.0	11.2	7.0
Benzaldehyde	1.5	0.0194	5.10	3.46	35.0	17.0	1.1
Amyl butyrate	1.3	0.0256	6.76	5.20	22.4	15.0	13.0
Delta dodecalactone	1.0	0.0015	0.40	0.40	90.0	7.0	7.0
Ethyl caprylate	0.72	0.0137	3.62	5.03	36.4	15.0	18.0
Vanillin	0.68	0.0256	6.76	9.94	86.0	4.5 [1]	12.2
Ethyl acetoacetate	0.52	0.0009	0.24	0.05	31.2	9.7	18.0
Ethyl butyrate	0.45	0.0086	2.27	5.04	15.2	16.0	33.0
Gamma octalactone	0.40	0.0029	0.77	1.93	59.4	10.3	26.0
Ethyl heptylate	0.17	0.0063	1.66	9.76	26.0	12.6	80.0
Isovaleric aldehyde	0.17	0.0021	0.55	3.24	7.2	14.0	82.0
Gamma undecalactone	0.15	0.0551	14.56	97.07	97.4	13.0	86.0
Ethyl valerate	0.094	0.0357	9.43	100.32	16.2	16.5	170.0
Gamma decalactone	0.09	0.0036	0.95	10.56	73.6	9.4	100.0
Ethyl cinnamate	0.016	0.0006	0.16	10.00	66.0	12.5	781.2
Heliotropine	0.0039	0.0015	0.40	102.56	78.4	3.8 [1]	1,830.0 [1]
Methyl ionine extra	0.0024	0.0009	0.24	100.00	60.0	9.0	3,700.0
Ionone alpha white	0.0004	0.0015	0.40	1,000.00	58.2	14.5	36,000.0

[1] These compounds were chromatogramed as saturated alcoholic solutions; in calculating the weighted peak heights, the solubility of the compounds in the alcoholic solutions was taken into account.

amount present in the finished beverage an estimate is furnished as to relative importance of each compound. The quotient was designated as the "unit flavor base." The larger the unit flavor base value, the more this component contributed to the flavor of the peach beverage. The unit flavor base values are listed in Table 3.

Since compounds detectable by gas chromatography may be volatile but not flavorful substances, gas chromatographic response needs to be related to flavor intensity. One technique for relating these two factors is "weighted peak height" (or peak area). This is obtained by dividing the peak height of a given compound by the threshold of that compound.

The procedure was suggested by Guadagni *et al.* (1966) who recommended that the peak areas be weighted by the threshold value in deciding upon the importance of each compound as a flavor substance. Peak height was used because the early peaks were needle-like and height could be measured better than area. The peaks with the longest retention times were flatter than the early peaks, thus for these peaks, peak height was not strictly related to concentration.

When the weighted peak heights and the unit flavor base values were transformed to logarithms, the relation between the two sets of values approached a straight line (Fig. 1). A highly significant correlation coefficient of 0.8691 was obtained. The unit flavor base and the weighted peak heights values are shown in Table 3.

Actually, the correlation coefficient between amounts of compounds in the beverage and gas chromatographic responses would likewise be correlated, for dividing by the threshold values merely introduced a constant factor. However, dividing by the respective threshold values weights each value according to its flavor potency.

Possible sub-threshold effects of isovaleric aldehyde, 2-methyl butyric acid, benzaldehyde, ethyl acetoacetate, ethyl cinnamate, and gamma undecalactone were studied. The combinations used and the sub-threshold levels expressed as a percentage of the threshold concentration are listed in Table 1. Table 2 shows the threshold levels for these compounds as well as the percent the sub-threshold amount was of the threshold.

Isovaleric aldehyde exhibited a sub-threshold effect with benzaldehyde and 2-methyl butyric acid when isovaleric aldehyde was at 33% of its threshold strength. Out of 19 combinations this was the only combination which showed a statistically significant sub-threshold effect.

To test for sub-threshold additive effects, ethyl acetoacetate and ethyl cinnamate at 92% and 1% of their respective threshold concentrations were dissolved in water and placed in a triangular test against distilled water. The panel could not detect a difference. Benzaldehyde was then added at 33% of its threshold level. The mixture of three compounds could not be distinguished from distilled water. This procedure was carried on until the mixture contained the eight compounds listed in Table 2. The order which was followed in adding compounds is the same as that listed in Table 2.

Upon incorporation of the eighth compound (amyl butyrate), the panel was still unable to select the chemical mixture from distilled water. Up to this point one might conclude that sub-threshold additives effects are not common.

A second series was then set up to investigate sub-

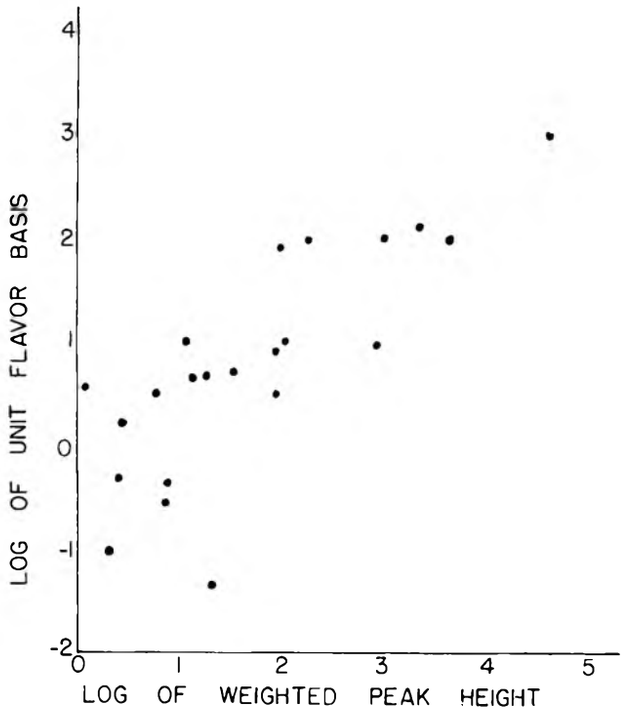

Fig. 1. Scatter diagram showing relation between log of flavor concentration/threshold value and log of gas chromatographic peak height/threshold value.

threshold additive effects in another way. Each of the eight compounds was incorporated in mixures at 80% of its respective threshold concentration. For this series, trials were started with the eight compounds in the mixture and, moving in reverse order, one compound at a time was dropped from the mixture. The panel could readily distinguish the mixtures from distilled water when there were three or more compounds in the mixture.

The first set of trials is perhaps more important than the second. In the first set, the range of concentrations is probably more typical of natural and formulated foods than the second. It would be rare to find all compounds at the same sub-threshold level. There is also the possibility that the panel members were selecting the test solution in the second method on the basis of sensitivity to one compound rather than the additive effect of eight compounds.

As explained in the Experimental section, the test solutions were varied by increments of 10 to establish the threshold concentration. The threshold level for each compound was selected as the point where the number of judges capable of selecting the correct sample barely exceeded the 5% level of probability.

Guadagni *et al.* (1963) set each of their sub-threshold concentrations so that the total for all the substances approximated the "theoretical" threshold. In our trials at the 80% sub-threshold levels, the "theoretical" threshold amount was exeeeded once two compounds were combined. Nawar *et al.* (1962) used the minimum concentration any member of their panel could detect, then set the concentration of each substance below the minimum (the exact amount is not specified); thus, the total for the proportionate part each compound contributed might have exceeded the 100% theoretical threshold.

A further factor relative to additive sub-threshold effects is that Nawar *et al.* (1962) were combining ketones as did Langer *et al.* (1964). Our results are more comparable to those of Guadagni *et al.* (1963) because we used substances from more than one class of compounds. In the first set of trials, the theoretical threshold concentration exceeded 100% after the third compound was added; yet we found no additive sub-threshold effect. In that respect our results differ from Guadagni *et al.* (1963).

One problem in establishing whether sub-threshold additive effects truly exist is the variability among judges in their sensitivity to different compounds. Blakeslee *et al.* (1935) found, for example, that the thresholds of 47 individuals for vanillin (one of the compounds we used) ranged from 1.56 to 6,400 ppm. Pangborn (1962) pointed out that even among highly-trained subjects there was a large amount of variation in scoring (though individual reproducibility was good) in testing for taste interaction of suprathreshold solutions of sucrose and NaCl.

Additive sub-threshold effects would seem to be logical, but proof is a very difficult matter. The more compounds one adds to the solution and the more judges there are, the greater the possibility becomes that the test solution is being selected because one judge is especially sensitive to one compound and a second judge to the same or a different compound. Lillard *et al.* (1961) reported that multiple correlations were higher for the compounds they studied, suggesting multiple effect of compounds rather than a single compound. Day *et al.* (1963) also concluded that as the number of compounds increased, the possibility of augmenting interactions increased.

The following six compounds were used to determine if the panel could distinguish a change in concentration of one compound in a mixture: ethyl cinnamate, gamma-undecalactone, benzyl alcohol, ethyl heptylate, amyl butyrate, and benzaldehyde. The compound being tested was varied 1% and 100% of beverage concentration.

In one series of trials the other five compounds of the mixture were at threshold level. For example, a basic solution was made up containing gamma-undecalactone, benzyl alcohol, ethyl heptylate, amyl butyrate, and benzaldehyde, each one of these compound being at 100% of its beverage strength. To part of the basic solution ethyl cinnamate was added at 1% of its beverage strength and to another part it was added at 100% of its beverage strength. The two samples were then compared in the regular triangular test. Another illustration is given in Table 4.

This same procedure was repeated until all six compounds had successively been the compound varied in conconcentration. Only the solutions containing 1% and 100% gamma-undecalactone with the other five compounds at threshold level were distinguished by the panel at 0.05 level of significance.

The reason the concentration of undecalactone caused a difference, when the other compounds did not, may have been a result of the concentration of undecalactone in the beverage. At the 100% beverage strength, undecalactone was well above the concentration of any other compound.

Table 4. Example of combinations used to test for concentration effect.

Series A (5 compounds at their beverage strength and the sixth substance [1] at 1% and 100% of its beverage strength)	
Ethyl cinnamate	0.16 ppm
Ethyl heptylate	1.66 ppm
Benzyl alcohol	2.75 ppm
Benzaldehyde	5.10 ppm
Amyl butyrate	6.76 ppm
Gamma undecalactone	14.56 ppm in one solution; 0.1456 in the other
Series B (5 compounds at their threshold levels and the sixth substance [2] at 1% and 100% of its beverage strength)	
Ethyl cinnamate	0.016 ppm
Ethyl heptylate	0.17 ppm
Amyl butyrate	1.3 ppm
Benzaldehyde	1.5 ppm
Benzyl alcohol	5.5 ppm
Gamma undecalactone	14.56 ppm in one solution; 0.1456 in the other

[1] Each of the six substances above was respectively adjusted to 1% and 100% of its beverage strength with the other compounds at their beverage strength forming the basic solution.

[2] Each of the six substances was respectively adjected to 1% and 100% of its beverage strength with the other compounds at their threshold levels forming the basic solution.

At 1% it was toward the lower end of the range of concentrations for the other compounds. When other compounds were added at 1% and 100% levels, no other compound exceeded the uppermost level of the five compounds held at 100% as drastically as did gamma-undecalactone.

The panel was capable of detecting a change in concentration of one substance among six only when the change represented a sizable portion of the total amount of material in solution. This conjecture is borne out by the fact that the panel was not able to distinguish between 1 and 100% gamma-undecalactone when the other five compounds were at beverage concentration.

The panel being unable to distinguish between the 1 and 100% solutions indicated that the threshold of a compound in distilled water may be different from the threshold in a mixture of compounds. For example, the threshold of benzaldehyde, one of the compounds used in these trials, was 33% of its beverage concentration. This would mean that 1% was below its threshold and 100% was above its threshold. If the threshold of benzaldehyde did not change when placed in a mixture the panel should have been able to distinguish between the solutions containing 1 and 100% benzaldehyde. Hinreiner *et al.* (1955b) observed that threshold levels varied according to the substrate.

The same mixture used to test for concentration effects was extracted with pentane, concentrated and injected into the gas chromatograph. By visual inspection of the chromatograms, one could distinguish between the solutions, but the solutions could be distinguished chemically only by concentrating their components.

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Organoleptic Identification of Roasted Beef, Veal, Lamb and Pork as Affected by Fat

SUMMARY—A taste panel was used to study the identification of roasted beef, pork, lamb and veal by flavor alone and the effect of fat on identification. Only about one-third of the panel could identify correctly all four meats by memory of the flavors. There was an increase in the total number of correct identifications made by comparison of the unknown roasted meat samples with known standards but this was not significantly greater than the total correct responses by memory of the flavor alone. Beef and lamb, but not pork and veal, were identified significantly less often when lean ground roasts were tested than when normal ground roasts (containing fat) were used. Texture, color, mouth feel, and other factors may be important in the identification of meat. Beef, lamb and pork fat, as well as these fats after extraction with chloroform:methanol, were added to lean veal prior to roasting. Addition of beef fat did not increase recognition of veal as beef. Pork fat contained a factor increasing identification of veal as pork, but this factor was water-soluble and could be removed. Lamb fat contained a component, or a fat-soluble component, that significantly increased the identification of veal as lamb.

INTRODUCTION

THE PROBLEM OF IDENTIFYING the chemical components of meat flavor could be simplified if it could be accepted that differences in meat flavors arise from the fat rather than the lean portion of the meat as has been reported (Hornstein *et al.*, 1960). However, such an assumption raises questions as to whether species flavor characteristics are really identifiable, and whether they could be attributed to the lean or fat portions of the meat.

Although many people believe they can identify cooked beef, pork, lamb or veal by flavor, preliminary organoleptic tests for the evaluation of meat flavor fractions indicated that the true differences between meats were not great. Without presenting data, Howe *et al.* (1937), commenting on the identification of meats by blindfolded people, indicated that beef and pork are identified correctly more often than lamb or veal. They attributed this to the presence of modified extractives and fat on the outside of the meat that has been exposed to high temperatures, and suggested that the distinguishable characteristics reside chiefly in a specific constituent of the fat.

Crocker (1948) prepared broths from meat and treated meats, and reported that neither the marrow, which is high in low-melting fats, nor the outside or tissue fats contribute greatly to beef flavor. Further studies by Kramlich *et al.* (1958) confirmed that the fat content of beef was not responsible for differences in flavors of broths prepared from meat. According to Hofstrand *et al.* (1960), lamb fat did not significantly affect the taste of broths made from lamb or mutton, but it may have contributed to the aroma of the broths.

The effect of fat on chicken flavor was reported by Peterson (1957) to be negligible. Extraction of lyophilized muscle with fat solvents did not reduce the flavor or odor of chicken broth prepared from the meat, and flavor profiles of the extracted meat were similar to, but weaker than, those of whole chicken broth. Pippen *et al.* (1954) also found that fat was of minor importance to chicken flavor, but did contribute something to the aroma.

The studies reported in this paper investigated the ability of the average consumer to identify meats commonly used and to determine some effects of the fat in characterizing the species of meat sources.

METHODS

THE MEATS USED in the study were eye roast of beef; veal shoulder roast; leg of lamb, rump end; and pork loin, loin end. These were purchased the day before the tests from a local commercial meat market. The meat was trimmed from the bone. When lean meat was desired, the meat was cut into small pieces, and all fat was removed including as much of the intermuscular fat as possible. Initial studies indicated that panel selections, using pieces of whole roast, were often based on color and texture rather than flavor. To reduce the influence of these extraneous factors the meat was passed twice through an electric grinder equipped with a plate with 3/16 in. holes.

When the effect of fat was to be evaluated, the meat was ground with 10% by weight of the selected fat to ensure homogeneous distribution. The ground meats were shaped into loaves and roasted at 350°F for 1½ hr to an internal temperature of approximately 190°. An initial test was conducted to compare the identification of hot roasts with that of cold roasts. The cold meat was identified with the same degree of accuracy as the warm meat; therefore, since it was more convenient to handle, all testing was carried out with meat roasted the afternoon before the test, refrigerated overnight and allowed to equilibrate to room temperature before serving.

The taste panel ranged from 27 to 31 people in the various experiments. A number of women were invited to participate on the panel to determine whether they were more discriminating than men in identifying the meats, based on their presumed familiarity with the flavor of the meats during preparation. Nonchemists, both men and women, were also included on the panel to establish the role of professional training in meat identification.

The panel was not a "trained" panel; it had not been screened to eliminate those with poor discrimination nor were the panelists specifically familiarized with the material to be tested. However, a number of the panelists had previously participated in organoleptic experiments with meat flavor and aroma. It was anticipated that a learning process might occur during the course of the testing but the panel responses to similar experiments near the beginning and end of the series were sufficiently alike to show this was not necessarily happening.

In the tests, the panelists were given four coded samples of meat in paper cups. The ground, roasted meat was broken into pieces to (1) reduce selection by color or texture, and (2) to mix the outer, brown crust with the inside meat for more uniform sampling. The panelists were asked to identify the meat samples, relying on their memory of the odor and taste of the meats. In one test the panelists were given four identified samples of meat and then requested to identify four unknown samples by direct comparison with the known meats.

The data were analyzed statistically by the Chi square test as described in Amerine *et al.* (1965). Analysis of variance could not be determined because the differences in sample sizes between the number of men and women and between the number of chemists and nonchemists were too great. Furthermore, the variance of the normal approximation is a function of sample size, so the variances of the groups would be quite different.

A number of the experimental results were confirmed with triangle tests where the panel selected the odd sample and identified both the odd and paired samples.

Beef, pork and lamb fats were extracted according to a modification of the method of Folch *et al.* (1957). Fifty-gram quantities of fat tissue were homogenized with 500 ml chloroform:methanol (2:1) for 1 min in a Waring blendor. The undissolved tissue and residue were removed by centrifugation. The chloroform:methanol solution was shaken with 100 ml of the upper phase of chloroform:methanol:water (8:4:3) mixture in a separatory funnel and the two phases allowed to separate. Water-soluble components in the upper phase were discarded. The chloroform:methanol solution was concentrated in a rotary evaporator under vacuum and the final traces of solvent removed by heating the fat to about 50°C on a water bath and bubbling N_2 through the liquid fat for 15 to 20 min.

Treatments

The meats were treated in the following manner. Experiments described in the paper refer to these treatments.

Experiment 1: Lean whole roasts. Whole roasts were trimmed of all visible covering fat, roasted and sliced for analysis.

Experiment 2: Lean ground roasts. Meat trimmed from the bones was cut into small pieces and all visible fat removed. After being ground twice, the meat was roasted.

Experiment 3: Natural ground roasts. Meat trimmed of a small amount of covering fat was ground twice and roasted.

Experiment 4: Direct comparison. Each meat was trimmed of fat, then fat was added back at the level of 10% of the weight of the meat. The meats were then ground twice and roasted. Labelled samples were presented to the panel. Unknown, coded samples from the same roasted loaves were given to the panel for identification by comparison with the labelled standards.

Experiment 5: Four veal samples. A veal shoulder roast, trimmed of fat, was ground twice and roasted. Each panel member received four coded samples of the roasted, ground veal in the same manner as in the previous experiments.

Experiment 6: Veal and beef, pork or lamb fat. Veal shoulder roast, trimmed of fat, was divided into four aliquots. Beef, pork or lamb fat, in 10% concentration by weight of the veal, was mixed with each of three aliquots of veal and ground twice before roasting. The fourth aliquot was prepared without added fat and served as a control for the identification of veal.

Experiment 7: Veal and extracted fat. The procedure was identical with that of Experiment 6 except that extracted fat was used. In all instances the term "fat" refers to fatty tissue rather than pure fats, except for the extracted fats.

RESULTS

THE RESULTS OF THE TESTS are shown in Tables 1 to 4. Table 1 is a compilation of the number and percentage of correct responses obtained in the various experiments. In Table 2 the experiments in each species of meat are arranged to show the relationship of significant differences among the response frequencies. There are no statistically significant differences among experiments that are underlined. The distribution of organoleptic responses to veal alone is presented in Table 3, and comparisons of the evaluations of veal to which the various fats have been added are shown in Table 4.

Effect of sex

No significant difference between the responses of men and women.

Table 1. Results of the experiments involving identification of four species of meat and the effect of fat on the rate of identification.

Expt No.[1]	Identification			Panelists					
					% Identifying samples correctly				
	Total No.	No. correct	% correct	Total No.	Beef	Lamb	Pork	Veal	All four
1	108	66	61.0	27	82.0	44.5	70.0	41.0	37
2	124	58	46.7	31	45.2	42.0	51.6	45.2	22.5
3	112	72	63.0	28	71.5	78.5	61.0	50.0	35.7
4	120	96	80.0	30	90.2	76.7	84.5	73.3	63.7
6	108	44	40.6	27	40.8	48.4	45.0	29.7	7.4
7	104	44	42.0	26	38.5	69.5	27.0	34.5	3.8

[1] For details of the experiments refer to methods.

Table 2. Relationship of statistical significance among the frequencies of correct responses as determined by Chi-square analysis.

	Experiment numbers[1]
Beef	4, 1, 3 — 2, 6, 7
Lamb	3, 4, 7 — 6, 1, 2
Pork	4, 1, 3 — 2, 6, 7
Veal	4, 1, 3 — 2, 6, 7

[1] Underlined experiments show no statistical differences among their responses.

Effect of training

There was no significant difference between the responses of chemists and non-chemists. (However, it should be noted that the number of non-chemists and of women on the panel were considerably less than the number of chemists and men.)

Effect of treatment

The only highly significant difference in responses of panelists was due to the treatments.

Effect of color and texture

The possible effect of the color and texture of the meat samples on identification can be seen by comparing the results of Experiments 1 and 2 in Tables 1 and 2. From Table 1 it can be seen that the percentage of correct identifications for lamb and veal on whole lean roasts is approximately the same as for ground lean roast, but the number of correct identifications of beef and pork decline for ground meat. However, according to Table 2, only the difference in the beef samples was statistically significant. Thus the texture of the whole lean beef roast, and possibly the color of the whole pork roast were sufficiently characteristic to aid in the identification of these meats.

Table 3. Identification of four identical samples of veal by 25 panelists.

Identification	%
Beef	32
Veal	33
Pork	24
Lamb	11

Correct identification

The percentage of the total number of correct identifications made in each experiment, and also the percentage of the panelists in each experiment that were able to identify correctly each meat sample and all four samples are shown in Table 1. The data based on all the identifications made indicate that, where the panelists depended on their memory of the characteristic flavor of the meat, the best they could do was to identify 63% correctly (Experiment 3). In Experiment 4, where the panelists had known meat samples as a basis of comparison, they identified 80% correctly.

The proportion of panelists correctly identifying all four meats by memory alone was 37% or less (column 10, Table 1); about two-thirds of the panel were able to do so when comparing the unknowns with known meat samples.

Although these data indicate that the ability of the panel to identify the meats by memory is not very high, statistical analysis of the correct identifications of the meats by variety shows no significant difference between memory identification and direct comparison with a standard. For this analysis the normal, ground meats of Experiment 3, identified from memory, were compared with the normal ground meats of Experiment 4, identified by direct comparison. The percent values (columns 6–9, Table1) show that all meats except lamb were identified less frequently by memory only, but the difference in the frequency of correct responses between Experiments 3 and 4 were not significant.

Effect of fat on meat flavor

It is popularly assumed that fat increases the flavor of meat. However, few organoleptic studies have been carried out to determine the role of fat in the formation of the characteristic flavor of the meats. A comparison of the results of Experiment 3, the normal ground meat, with Experiment 2, the lean ground meat, indicates that the absence of fat led to a significant decrease in the number of correct identifications of beef and lamb. The identification of veal and pork was not influenced by the absence or presence of fat.

Hornstein *et al.* (1960) stated that lean meats of the various species have essentially the same basic aroma and that the species-specific aroma is due to the fat. If the flavor precursors in the fat form the characteristic aroma on heating, the identity of the lean meat may not be important.

Table 4. Effect of adding unextracted or extracted beef, pork or lamb fat to veal on identification compared with that of veal alone or authentic meat.

	Comparison with			
	Veal[1]		Authentic meat[2]	
Added fat	Unextracted fat	Extracted fat	Unextracted fat	Extracted fat
Beef	No. sig. difference	No. sig. difference	Sig. lower	Sig. lower
Pork	Sig. higher	No. sig. difference	No. sig. difference	Sig. lower
Lamb	Sig. higher	Sig. higher	Sig. lower	No. sig. difference

[1] Comparison of identification of veal and added fat with random identification of veal as the particular meat (Expt. 5).

[2] Comparison of identification of veal and added fat with identification of natural ground roast (Expt. 3).

On the other hand, if lean meat is the major contributor to the characteristic flavor note, then, fat, regardless of species, could play a minor role.

To explore this further, veal was selected as the basic lean meat to which fats of other species were added. The choice of veal was made on the basis of the natural leanness of the meat and its normally bland flavor. In Experiment 5, four samples of veal were given to each of 25 panelists. The identifications are shown in Table 3. Of the 100 samples of roasted ground veal presented, about one-third were identified as beef and one-third as veal; pork was the choice of 24% of the panel and lamb, 11%. Beef, pork or lamb fat, in portions equal to 10% of the weight of the veal, were ground with veal and roasted. Identification was considered correct when the panelist recognized the veal plus fat as the species of meat corresponding to the fat. A scoring of "veal" for a sample of veal plus fat was considered incorrect. Experiment 6 in Table 1 shows the results obtained. The Chi-square test was used to analyze the difference between the responses to veal alone as a specific species of meat (Experiment 5) and the responses to the veal plus fat as the same species. The addition of beef fat did not significantly increase the number of identifications of veal as beef. Addition of pork fat resulted in a number of correct identifications of veal as pork that was significant ($P < 0.05$), while the identification of veal + lamb fat as lamb was highly significant ($P < 0.01$).

The responses in the identifications of veal + fats (Experiment 6) also were compared with those of natural ground roast (Experiment 3) as shown in Table 2 to determine whether the rate of identifications was the same. The identification of both "beef" and "lamb" was significantly less when the fats were added to the veal, but the addition of pork fat to veal resulted in a rate of identification that was not significantly different from that of the natural meat.

Fat was extracted from fatty tissue and added to the veal under the same conditions as the previous experiment. The results are shown as Experiment 7 in Table 1. A comparison of the results of the unextracted fat and the extracted fat experiments (Experiment 6 vs Experiment 7) indicated no effect of fat extraction on the identification of veal as beef, but the identification of pork was less frequent while the number of correct identifications of lamb increased. Chi-square analysis of the differences between the frequency of responses in these tests indicates that now beef and pork were not identified to a significantly different extent than veal was when presented alone (Experiment 5).

Lamb identification, however, increased significantly. From Experiments 3 and 7 it can be seen that veal with the addition of extracted beef or pork fat was identified significantly less often than natural beef or pork, but veal with lamb fat was identified as lamb approximately as often as natural lamb. These results are summarized in Table 4.

DISCUSSION

THE CORRECT IDENTIFICATION of meat by memory of flavor alone is not as readily accomplished as it appears to the average person. Before beginning the tests, most of the panel felt they could identify the various species of meat; however, only about one-third of the group was able to identify all four meats correctly. The effect of texture and color of the meat, as well as the method of preparation—the seasoning, spicing and garnishing—influences the psychological processes leading to recognition. When identified standards were supplied for comparison, the number of correct identifications of the meats increased but were not statistically significant; about a third of the panel still was unable to identify all four of the meats.

While the ability of either men or women to identify the meats correctly was not considered an inheritable, sex-linked characteristic, it was thought that women, by virtue of their experience and training in food preparation, might score better than men in this type of test. The results, however, indicate no statistically significant difference between the sexes. Minnich *et al.* (1966) however, reported that men consistently displayed greater taste sensitivity than women in the identification of chicken flavor in broth.

The effect of texture, color and other extraneous factors must be eliminated from tests of this type. Our results confirm the findings of Howe *et al.* (1937) that beef and pork were identified correctly more often than lamb or veal. These authors attributed the differences to the effect of fat whereas our data, using crumbled ground meat, implicated selection based on the color and texture of the meat. Fat, however, may play a role in the identification of meat; beef and lamb were identified significantly more often in fat-containing ground roasts than in lean ground roasts, whereas the identification of veal and pork were unaffected by the presence of the fats.

Another type of experiment in which specific fats were added to lean meat was carried out to determine the role of the fat in characterizing the meat species. There are three possibilities for the development of flavor involving fat: (1) the fat contains precursors that liberate the species-specific aroma on heating, and the lean meat itself has little or no effect; (2) there is an interaction between lean meat and fat components to give the desired aroma, and the lean meat components may be either specific or non-specific for the species; (3) fat does not contain compounds that yield species-specific aroma.

Veal was selected as the basic lean meat because it has less fat and its flavor is blander and less distinctive than the other meats. The flavor of veal resembles that of beef resulting in confusion between the two, and this was demonstrated when about a third of the panel identified veal as beef and another third correctly called it veal.

It was surprising to find 25% of the panel identifying veal as pork. Beef fat added to veal did not make veal resemble the normal beef roast and the identification of veal plus beef fat as "beef" was about the same as the random identification of veal alone as "beef." Pork fat, however, contained a factor that increased recognition of veal as pork to a point where it was significantly like that of normal pork. Extracting the fat with fat-solvents and washing with water removed this factor and then pork fat did not affect the identification of veal. The results with lamb fat are of interest. The addition of lamb fat significantly increased recognition of veal as lamb, but not to the extent to which normal lamb was identified. Extracting the fat resulted in the concentration of the flavor factors

so the veal was identified as lamb as often as regular lamb.

Hofstrand *et al.* (1960) studied the effect of fat on the flavor of lamb and mutton. Depot fats were found to have flavor components that were changed by heat; however, heating the fats gave aromas that were not particularly lamb-like according to the judges. Furthermore, the aroma fraction was water-soluble, whereas the factor in the study reported here was concentrated in or with the fat.

The high rate of lamb identification was surprising because most of the panelists had indicated that they had eaten very little lamb previously or that they did not care for lamb. The flavor of lamb is evidently so characteristic it can be identified by people with little previous exposure.

On the basis of these experiments the role of fat in giving character to the flavor of a meat is not the same in every case. Beef fat appeared to have little or no effect on the development of a characteristic beef aroma. Pork and lamb fats apparently contain some factor(s) that develop a specific aroma on heating with veal. The pork fat factor, however, is water-soluble, while the lamb fat factor is either a component of the fat or is fat-soluble.

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Texture Profile of Ripening Pears

SUMMARY—Pears of the Ovid variety were harvested at the normal stage of maturity, and ripened in a 70°F storage room. Samples of the fruit were taken out at regular intervals for texture measurements which consisted of the Magness-Taylor pressure test and a modified General Foods Texture Profile. The adhesiveness of the pears was zero. The viscosity of the whole fruit could not be measured. All other parameters of the G. F. Texture Profile (hardness, cohesiveness, elasticity, brittleness, chewiness, and gumminess) decreased during ripening at approximately the same rate as the pressure test. It is suggested that this characteristic of ripening pears is responsible for the success of a simple single measurement (the puncture test) for measuring the complex phenomenon that is called "textural quality" of pears.

INTRODUCTION

FOOD RHEOLOGISTS might be divided into two hypothetical classes. The first consists of those who consider texture a fairly simple property of food that should be subject to precise measurement rather easily. This class can, for example, point to the success of the Magness-Taylor pressure tester in measuring the maturity of pears and the Tenderometer in measuring the maturity of fresh green peas to support their view. However, this class is faced with the problem of explaining the great difficulty that has been encountered in measuring the texture of a large number of foods.

The second class consists of those who consider that texture is the sum of a number of different properties of foods that are sensed by several different organs of the human body. Some of these properties may be complex in nature. To support their view this class can point to the seeming resistance of many foods to satisfactory objective measurement of their texture by simple apparatus. This class is faced with the problem of explaining the ease with which comparatively simple instruments measure the texture of certain foods.

The Magness-Taylor pressure tester (Magness *et al.*, 1925) is an example of a simple texture-measuring instrument. It appears to be routinely used to choose the time to harvest and process pears. Salunkhe *et al.* (1966) state, "It has been agreed that the pressure tester is the best single test for pear maturity as the pear is not ready to eat when picked," and they note that recommendations range from 15 to 20 pounds pressure test at harvest time. A group in California (Leonard *et al.*, 1954; Luh *et al.*, 1955; Dame *et al.*, 1956; Leonard *et al.*, 1957; and Claypool *et al.*, 1958) have shown in a series of studies that a pressure test of 2 to 3 pounds gives the best overall quality in canned pears and that using fruit with a higher or lower pressure test than this range causes a loss of quality in either texture, flavor, aroma or appearance.

The General Foods Texturometer (Friedman *et al.*,

1963) is an example of a complex texture measuring instrument from which the G.F. Texture Profile is obtained. This consists of the parameters hardness, cohesiveness, elasticity, adhesiveness, brittleness, chewiness, gumminess and viscosity. There is no published data describing how the various parameters of the G.F. Texture Profile of pears change as the pears ripen. This study was designed to find the relation between G.F. Texture Profile and the Magness-Taylor pressure of ripening pears and to throw some light on the conflict between simple texture vs. complex texture theory of foods.

EXPERIMENTAL

Preparation of fruit

Ovid variety pears were obtained from the Experiment Station orchards. The Ovid pear is a Bartlett seedling that matures about four weeks later than the regular Bartlett pear. The texture of its flesh closely resembles that of the Bartlett. The pears were stored at 32°F for six weeks, and then placed in a ripening room at 70°F. A sample of 25 pears was taken three times weekly for texture measurements. Each pear was punctured twice with a 7/16" diam. Magness-Taylor pressure tip mounted in an Instron universal testing machine. The 7/16" diam. pressure tip was used instead of the usual 5/16" diam. tip because it came closer to the diameter of sample used in the G.F. Texture Profile.

The adaptation of the Instron machine for food work has been described by Bourne *et al.* (1966). Two 2-cm diam. cylinders of tissue were cut from each pear at right angles to the core axis with a cork borer and trimmed to a height of 1 cm. These cylinders were used for the modified G.F. Texture Profile test. Both Magness-Taylor and G.F. Texture Profile were performed on the same pears. All tests were made with the skin removed from the test section. Mean values were calculated from the 50 readings for each parameter made on each test day.

Use of Instron machine

The Instron machine was used to perform the G.F. Texture Profile. A flat horizontal plate, approximately 15 cm diameter, was attached to the inverted load cell which was bolted to the moving crosshead. The crosshead was set to cycle with a vertical reciprocating movement at a constant speed of 5 cm per min and with a stroke length of 7.5 mm. The maximum space between the moving horizontal plate and the stationary horizontal bedplate of the machine was 10 mm and the minimum space was 2.5 mm. The moving plate compresses the 10 mm high cylinder of pear flesh down to 2.5 mm high (the first "bite") then it rises back to its starting position and moves down to compress the pear tissue down to 2.5 mm again (the second "bite"). Each piece of fruit was subjected to two "bites." The chart was driven at 50 cm/min, exactly 10 times the speed of the moving crosshead. This gave a tenfold magnification of the distance axis and opened out the force-distance curve so that the various measurements required for the G.F. Texture Profile could be readily obtained.

There are some differences between performing the G.F. Texture Profile Test on the G.F. Texturometer and performing it on the Instron machine. These differences make at least one qualitative change in the G.F. Texture Profile. They probably cause some quantitative changes as well. Because the G.F. Texturometer is driven by an eccentric, the speed of travel of the moving parts varies in a sinusoidal pattern. The G.F. Texturometer is decelerating as it approaches the end of the compression stroke and it slowly accelerates again as it begins its upward stroke. The speed is at a maximum at the center position of each stroke.

Because the working parts of the G.F. Texturometer are driven by a lever moving about a fulcrum, the plunger moves through the arc of a circle. One edge of the plunger contacts the food at first and as the downstroke continues, the area of plunger pressing on the food increases until the entire plunger area is in contact with the food at the end of the downstroke. During the upstroke the contact area between the plunger and the food becomes progressively smaller. As a result, the force-time curves from the G.F. Texturometer have rounded force peaks.

In contrast, the speed of the Instron is constant at all times. The Instron approaches the end of the compression stroke at constant speed and deceleration is extremely rapid. When the compression stroke is completed, it abruptly reverses direction and starts its upward stroke at full speed.

This abrupt change in direction at constant speed results in sharp peaks in the force-distance curve (see Fig. 1). Since the compressing plunger moves in a strictly rectilinear manner the plunger area in contact with the food is constant during the test (unless the food spreads out under compression).

The chart on the G.F. Texturometer gives a force-time curve, and the areas under the curves A_1 and A_2 are therefore force-time integrals. The chart of the Instron gives a force-time curve also, but since both chart and drive motor are synchronously driven this is also a force-distance curve. Hence the areas under the Instron curves are both force-time and force-distance integrals. Since the work function is a force-distance integral, the areas under

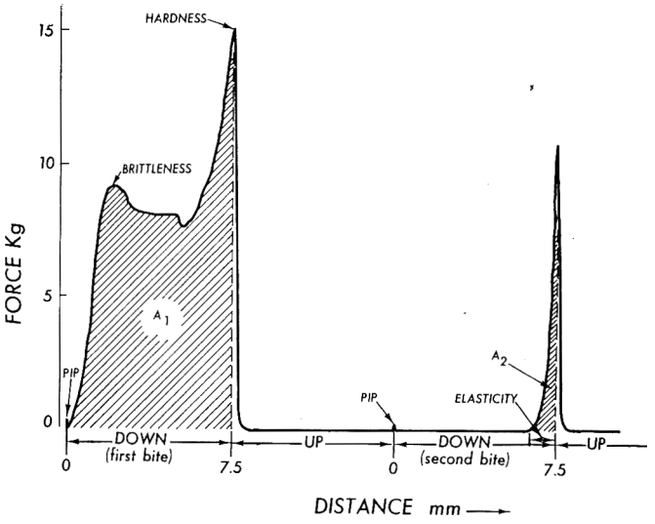

Fig. 1. Direct trace (heavy line) of force-distance curve obtained for a G.F. Texture Profile on a cylinder of pear tissue in the Instron machine. The test consists of two complete compression-decompression cycles.

the Instron curves are true measurements of work and can be used as such.

Interpretation of curve

Fig. 1 is a direct tracing of a force-distance curve drawn by the Instron chart for one cylinder of pear tissue. The tracing is reproduced here as the mirror-image in order that the distance axis read from left to right in the conventional manner. The chart was set to make a "pip" every time the crosshead commenced a down stroke. As the crosshead starts a pip is marked on the chart. The force then rises steadily until there is a major break in the fruit tissue denoted by a drop in force. The G.F. Texture Profile defines the height of this peak as "brittleness." It is a force measurement and has the dimension $m\ l\ t^{-2}$. The pear tissue continues to crack open and squash flat as the downstroke continues. The squashing, which requires increasing force, continues until the downstroke ends and the upstroke begins. The maximum height in this part of the curve is defined as "hardness" by the G.F. Texture Profile. It is another force measurement with dimensions $m\ l\ t^{-2}$.

As the return stroke begins, the force drops off to zero quickly and most of the upstroke occurs with zero force. When the upstroke is completed the machine automatically reverses direction and immediately begins the second downstroke. The marker on the chart records a small "pip" to mark the onset of the second downstroke. The first section of the second downstroke occurs at zero force because the pear does not recover its full height. When the compressing plate contacts the fruit for the second time, the force again rises in a smooth curve with no breaks until the stroke is completed. At this time the machine again abruptly reverses direction, the force falls back quickly to zero and the second stroke ends uneventfully.

"Elasticity," as defined in the G.F. Texture Profile, is the distance that the food recovers between the first and second bites. With the Instron machine this is easily measured by dropping a perpendicular from the peak of the second bite and measuring the distance along the baseline from this point back to the point where the compressing plate contacted the pear. With the G.F. Texturometer this measurement approximates the distance of recovery. With the Instron machine this is an exact measure of the distance and has the dimension l.

The area between the force curve and the left hand side of the perpendicular drawn on the second bite is a measure of the work done on the pear by the machine during the second bite. The area between the right hand side of this perpendicular and the force curve on the second bite is a measure of the work done on the machine by the pear as it recovers from the compression This work is being returned to the machine. Similarly, on the first bite, the area under the curve during the downstroke (to the left of the perpendicular) measures the work done on the pear by the machine; the area under the curve on the upstroke (to the right of the perpendicular) measures the work done on the machine by the pear.

Because these two sections under the curve can be separated so easily on the Instron chart, it seemed preferable to measure areas A_1 and A_2 as the work done on the pear by the machine and exclude the smaller areas to the right of the perpendicular lines. In the G.F. Texturometer the total area under each curve is measured and there is no separation of work done during compression from work returned during decompression. Cohesiveness, as defined by the G.F. Texture Profile, is the ratio of the two total areas under the curve. In this paper cohesiveness is the ratio of the two areas to the left of the perpendiculars,

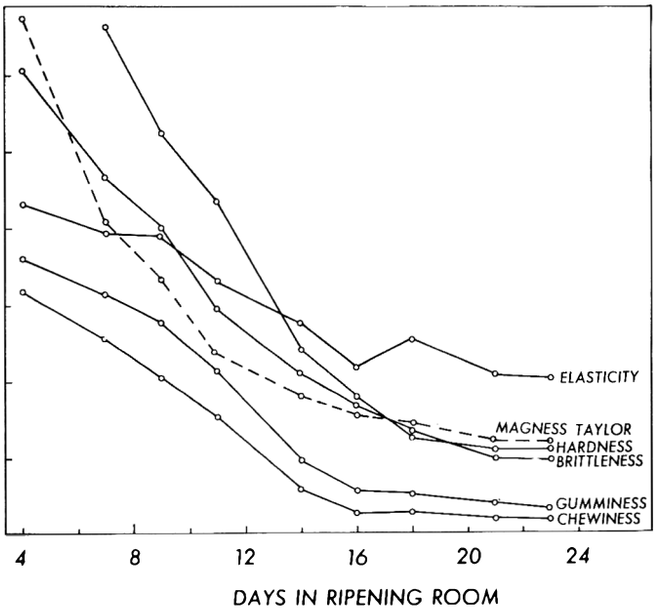

Fig. 2. Changes in G.F. Texture Profile and Magness-Taylor puncture test on pears as a function of time in 70°F ripening room. All scales up the ordinate are linear and commence at zero. Full scale values are: brittleness, hardness and Magness-Taylor, 14.0 Kg; chewiness 2.80 Kg mm; elasticity 2.80 mm; gumminess 1.4 Kg.

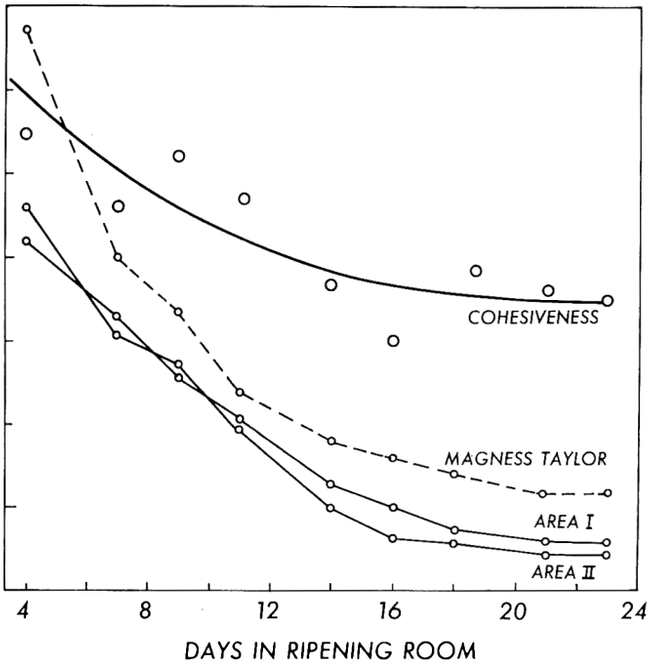

Fig. 3. Changes in A_1, A_2 and cohesiveness of pears as a function of time in 70°F ripening room. All scales up the ordinate are linear and commence at zero. Full scale values are: A_1 280 cm², A_2 14cm², cohesiveness 0.07, Magness-Taylor 14.0 Kg.

A_2/A_1, and it is dimensionless. The areas were measured by planimeter. The areas under the curves to the right of the perpendiculars are quite small compared to the total areas and they probably would not change the cohesiveness values significantly had they been included.

The G.F. Texture Profile defines chewiness as the product of hardness × cohesiveness × elasticity; gumminess is defined as the product of hardness × cohesiveness. These two calculations have been faithfully followed in this paper, using the values obtained for hardness, cohesiveness and elasticity as described above. Under these conditions chewiness becomes a work function with dimensions $m\ l^2\ t^{-2}$ and gumminess a force function with dimensions $m\ l\ t^{-2}$.

RESULTS AND DISCUSSION

THE G.F. PARAMETER OF VISCOSITY is not used since pears are solid. The parameter of adhesiveness was vanishingly small on all samples. Adhesiveness can therefore be considered as zero in pears at all stages of ripeness.

In Fig. 2 the changes in the Magness-Taylor reading, and in the G.F. Texture Profile parameters of elasticity, hardness, brittleness, gumminess and chewiness are plotted as a function of days in the ripening room. The striking feature of these data is that all these parameters of the G.F. Texture Profile run approximately parallel to each other and to the Magness-Taylor test. There is an initial rapid decrease in value of each parameter which lasts for about 16 days and thereafter a slow decrease in each parameter. A measurement of any one of these parameters would be an excellent index of any of the other parameters.

In common with the parameters shown in Fig. 2, the parameter of cohesiveness decreases during the first two weeks in the ripening room and then flattens out. However, cohesiveness does not decrease as much proportionately as the other parameters and there is much greater scatter in the experimental points. An examination of the sources of the cohesiveness value shows why this happens.

Cohesiveness is defined as the quotient: area AII/area AI. These two areas are plotted in Fig. 3. They both decrease in the same manner and at about the same rate as the Magness-Taylor reading and the other G.F. Texture Profile parameters. Since AI is about 20 times as large as AII, cohesiveness is numerically small. Small fluctuations in AI and AII appear as larger fluctuations in the cohesiveness value, especially when the fluctuations in AI and AII reinforce or oppose one another. Having taken these difficulties into account, the G.F. Texture Profile parameter of cohesiveness runs approximately parallel with the other parameters and with the Magness-Taylor reading.

CONCLUSIONS

THE EVIDENCE SHOWS how well all the applicable texture parameters of pears decrease in unison during ripening. At first the rate of decrease is rapid, but after about 2 weeks the rate of change slows down for all parameters. With this kind of behavior, almost any single texture measurement will give a good index of the ripening of the pear.

The data also throw some light on the simple texture vs complex texture controversy. The author interprets the evidence as meaning that the texture of pears is indeed a complex thing, but it appears to be simple because all the parameters run parallel as the pears ripen. When all the various textural parameters of a food change together in the same direction and at the same rate, the texture measurement of that food appears to be simple. When the parameters do not change together or change in different directions, the texture of that food would appear to be complex. In all cases however, texture is in fact a complex property of food.

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